DISSERTATION

DRUG EFFLUX SYSTEMS AND ANTIBIOTIC RESISTANCE IN *Burkholderia pseudomallei*

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fort Collins, Colorado

Summer 2009

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May 1, 2009

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LILY TRUNCK ENTITLED "DRUG EFFLUX SYSTEMS AND ANTIBIOTIC RESISTANCE IN *Burkholderia pseudomallei*" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

DRUG EFFLUX SYSTEMS AND ANTIBIOTIC RESISTANCE IN *Burkholderia pseudomallei*

Burkholderia pseudomallei, the etiologic agent of melioidosis, is intrinsically resistant to most antibiotics. A predicted 10 RND efflux transporters are encoded by the *B. pseudomallei* genome; 3 have been characterized (AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC) as major contributors to the intrinsic aminoglycoside, macrolide, chloramphenicol, and trimethoprim resistance of this organism. AmrAB-OprA is constitutively expressed in most strains and confers resistance to aminoglycosides. Gentamicin susceptible clinical isolates have been identified and work in this dissertation demonstrates that such susceptibility occurs as a result of either insufficient expression or deletion of *amrAB-oprA*. The mechanisms regulating expression in these strains are unclear, but are not related to mutations in this operon's putative repressor (AmrR) or mutations in the regulatory regions of *amrAB-oprA*. Expression analysis of seven *B. pseudomallei* RND efflux pumps (*amrB, bpeB, bpeF, bpeH, BPSL0309, BPSL1267,* and *BPSL1567*) in 60 clinical and environmental *B. pseudomallei* isolates from Thailand demonstrated (i) efflux pump expression is prevalent in both clinical and environmental strains (ii) *bpeH* is expressed at a higher level in clinical isolates as compared to environmental isolates and (iii) efflux pump expression correlates with

resistance/susceptibility to several antibiotics. These data suggest possible substrates for uncharacterized efflux pumps or, alternatively, co-regulation of resistance determinants. Since a deficit in efflux resulted in susceptibility to otherwise clinically useful drugs and that expression of efflux pumps was prevalent in *B. pseudomallei,* we suggest that efflux pump inhibitors would broaden the spectrum of antibiotics useful for treatment of melioidosis. To facilitate discovery and characterization of such compounds, we have developed a panel of efflux deficient *B. thailandensis* strains that express the *B. pseudomallei* efflux pumps, *amrAB-oprA* and *bpeAB-oprB*, which can be handled under BSL2 conditions. When expressed in the surrogate background, these pumps have the same substrate profile and can be inhibited by clinically insignificant efflux pump inhibitors to a degree similar to that observed in the native background. In summary, we have assessed the contribution of efflux to antibiotic resistance in *B. pseudomallei* and described a tool for discovery and characterization of efflux pump inhibitors for pumps expressed in this organism.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my advisor, Dr. Herbert Schweizer, whose mentorship and patience have facilitated not only the work presented in this dissertation but also my personal development as a scientist. During the course of my graduate work, Dr. Schweizer has been an ever-present source of encouragement and advice; for that I am endlessly thankful. I would also like to express my appreciation to the members of my graduate committee, Drs. Steven Dow, Richard Slayden, and Laurie Stargell, whose guidance ensured the continual and fruitful progress of my scientific endeavors.

Dr. Ayush Kumar deserves my special thanks, as the technical support and advice he shared with me during the early stages of my graduate career helped me to develop the foundations which supported the rest of my graduate work. I would also like to thank the other members of the Schweizer Lab (past and present) including but not limited to: Heather Blair, Dr. Kyoung-Hee Choi, Dr. Carolina Lopez, Dr. Takehiko Mima, Nicole Podnecky, Katy Quinn, and Drew Rholl whose support, humor, and camaraderie made working in the lab a truly enjoyable experience.

Lastly I would like to thank my family and friends whose support and understanding have made the hard times less of a struggle. I am especially thankful for the unwavering encouragement from my parents. Ashley Beggs, Sam Lyons, Jenni Mullins, and Annah Rolig are due my eternal gratitude for their friendship and support.

Dedicated to

my parents

for their relentless encouragement

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LIST OF ABBREVIATIONS

A_{600nm}	absorbance at 600 nm
ABC	ATP binding cassette superfamily
AC	acriflavine
AG	aminoglycosides
Ap	ampicillin
ATP	adenosine triphosphate
B.	*Burkholderia*
BL	β-lactams
bp(s)	base pair(s)
BS	bile salts
BSL	biosafety level
C	Celsius
C.	*Caenorhabditis*
CDC	Centers for Disease Control
cDNA	complementary DNA
cfu	colony forming units
CB	carbenicllin
GC	cytosine guanine
Cla or Clr	clarithromycin
Cli or Cld	clindamycin
Cm or CL	chloramphenicol
CP	cephalosporins

CPZ	chlorpromazine
CT	cefotaxime
C_t	threshold cycle
CV	crystal violet
DAP	diaminopimelic acid
Dc	deoxylcholate
DC	doxycycline
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
e.g.	for example
EB	ethidium bromide
ELISA	enzyme-linked immunosorbantassay
EM or Ery	erythromycin
EPI	efflux pump inhibitor
et al.	and the others
FA	fatty acids
FBI	Federal Bureau of Investigation
FQ	fluoroquinolones
FU	fusidic acid
g	gram
Gm	gentamicin
h	hour
HSL	homoserine lactone
i.e.	id est- that is
i.n.	intranasal
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
Km	kanamycin

L	liter
LB	Luria-Bertani
LD	lethal dose
LE	levofloxacin
LN	lincosamide
LPS	lippopolysaccharide
LSD	least significant difference
LSLB	low salt Luria-Bertani
MATE	multi-drug and toxic compound extrusion family
MC	mitomycin
MFP	membrane fusion protein
MFS	major facilitator superfamily
mg	milligrams
MHA	Muller-Hinton agar
MIC	minimum inhibitory concentration
ML	macrolides
ml	milliliter
MLS	macrolide-lincosamide-streptogramin
mM	millimolar
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NA	naladixic acid
ND	not done
NF	norfloxacin
NI	not identified
nm	nanometer
NO	novobiocin

Nor	norfloxacin
OMP	outer membrane protein
OS	organic solvent
P.	*Pseudomonas*
PB	polymixin B
PBP(s)	penicillin binding protein(s)
PCR	polymerase chain reaction
PLC	phospholipase C
PMZ	promazine
PR	protamine
P_{tac}	*E. coli lac/trp* operon hybrid promoter
PU	puromycin
Q	quinolones
QRDR	quinolone resistance determining region
*q*RT-PCR	quantitative real-time PCR
r	resistance
RF	rifampicin
RNA	ribonucleic acid
RND	resistance-nodulation cell-division
rpm	rotations per minute
SCV	small colony varients
SDS	sodium dodecyl sulfate
SF	sulfonamides
SML	small molecule libraries
SMR	small multidrug resistance family
Spc	spectinomycin
Str	streptomycin
TC	tetracyclines

TM	trimethoprim
TMS	transmembrane segments
TR	triclosan
TS	co-trimoxazole
TTSS	type three secretion system
TX	triton 100
TZ or Cef	ceftazidime
USDA	United States Department of Agriculture
VBNC	viable but non-culturable
x-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
XL	co-amoxiclav
Zeo	zeocin
μm	micrometers
μg	micrograms
μl	microliters

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CHAPTER 1

Burkholderia pseudomallei AND MELIOIDOSIS

1.1 Microbiology of *B. pseudomallei*

Burkholderia pseudomallei is a Gram-negative, aerobic saprophyte (58). The bacterium can also grow anaerobically in the presence of nitrate or arginine. This and other important microbiological characteristics are summarized in **Table 1-1**. Measuring approximately 0.8 x 1.5 μm, *B. pseudomallei* is a relatively small bacillus. *B. pseudomallei* is non-spore forming, however, it is able to survive in harsh environmental conditions as well as nutrient deficient conditions, such as distilled water (58, 119, 144). The optimal growth temperature for *B. pseudomallei* is 37°C; at 42°C the bacterium grows well in liquid culture until the nutrient source is limited, at which point ~80% of the culture becomes viable but non-culturable (VBNC) (58). After 24 hours at 0-4°C viability is decreased >90%, however, long-term storage at -80°C is possible in 15% glycerol (58).

There are seven colony morphologies (Type I-VII) associated with *B. pseudomallei* ranging from a fried egg appearance to large mucoid colonies. Type I (dry rough colonies with a fried egg appearance) is the most common morphology, however, one strain can manifest multiple phenotypes, sometimes simultaneously (10). Types II-VII are found in clinical specimens but are rarely cultured from the soil, indicating that phenotype switching may have some biological relevance (10).

Table 1-1 Microbiological Characteristics of *B. pseudomallei* [adapted from (7, 84)]

Characteristic or Test	Result
Gram Stain	Gram (-) with bipolar staining
Oxidase	+
Motility	+
Oxidation of:	+
Glucose	+
Xylose	+
Maltose	+
Adonitol	+
Sucrose	+
Lactose	+
Arabinose	-
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Hydrolysis of:	
Gelatin	+
Esculin	+
Urea	+
ONPG	-
Growth on:	
BCSA	+
MacConkey	+
42°C	+
Citrate Agar	+
Nitrate reduction	+
with gas production	+
Resistance:	
Polymyxin B 300 μg/mL	+
Penicillin 10 μg/mL	+
Gentamicin 30 μg/mL	+

1.2 Sequence Analysis

In 2004, the first complete genome of *B. pseudomallei* (strain K96243) was described providing researchers with a key tool for investigation of the organism (53). To date, there are 4 complete and 17 unfinished *B. pseudomallei* sequences available from Genbank. Sequencing of the 7.25 megabase pair genome of K96243 confirmed two chromosomes (which are circular replicons) of 4.07 megabase pairs and 3.17 megabase pairs; with the larger chromosome (chromosome 1) encoding functions associated with central metabolic processes and cell growth and the smaller chromosome

(chromosome 2) encoding for accessory functions (53). In fact, a group analyzing the transcriptome of *B. pseudomallei* across all growth phases found that early-phase gene expression was primarily located on chromosome 1 while stationary-phase gene expression was biased to chromosome 2 (105). A study using comparative genomic hybridization to compare 100 strains of *B. pseudomallei* to reference strain K96243 found that 86% of the genome was stable across all strains; this portion of the genome was thus named the "core genome" (108). The remaining 14% was highly variable across the 100 strains tested and was named the "accessory genome" (108).

The genome variably contains at least 16 genomic islands (distinguished from the rest of the genome by GC content), which comprise 7.6% of the larger chromosome and 4.2% of the smaller chromosome in K96243, indicating that horizontal acquisition of DNA was important to the evolution of *B. pseudomallei* (53). This hypothesis is further supported by evidence that the genomic islands contain coding sequences that are similar to mobile genetic elements such as bacteriophage and transposons (53). One of the genomic islands appears to be an integrated plasmid that contains coding sequences for recombinase, conjugal plasmid transfer, and replication proteins (53). However, it is important to note that there seems to be some genetic fluidity between strains of *B. pseudomallei*; two of the genomic islands are part of the core genome while the other 14 are considered part of the accessory genome (there may also be additional islands that have yet to be sequenced from other strains) (108). The genomic sequence has led to important discoveries in the virulence, pathogenesis, immune response, diagnosis, and antibiotic resistance of *B. pseudomallei,* as well as aided in the phylogenetic organization of the genus.

1.3 Phylogeny

The genus *Burkholderia* contains over 30 diverse species. Largely comprised of non-pathogenic soil bacteria, the genus does contain some important plant and animal pathogens. The *recA* based phylogenetic tree in **Figure 1-1** illustrates the relationship between all the *Burkholderia* species in addition to some other closely related organisms. *B. mallei* and *B. pseudomallei* are closely related while *B. cepacia* has a more distant relationship to the other two clinically important members of the genus. Sequence similarities indicate that *B. mallei* is a clone of *B. pseudomallei* that has undergone genomic reduction to become its own species (54). Loss of large chromosomal segments in *B. mallei* as compared to *B. pseudomallei* has been illustrated by DNA microarray analysis (91). Further support for this conclusion comes from the fact that *B. mallei* only has one of the genetic islands found in *B. pseudomallei* and, although it appears that the genomic islands arose due to horizontal gene transfer, it is unlikely that the transfer has occurred since the divergence of *B. mallei* from *B. pseudomallei* (53, 83). Thus, it is likely that gene loss was a major contributor to the evolution of *B. mallei* while horizontal transfer was more important to the evolution of *B. pseudomallei.*

Interestingly, *B. thailandensis,* a close relative to *B. pseudomallei* is not pathogenic. Some major differences between the two bacteria include 16S rRNA sequence differences, ability to assimilate arabinose and ethanol, and their secreted protein profiles (83). Moore et al. suggest that the ability to assimilate arabinose, a characteristic of *B. thailandensis*, is an antivirulence factor and that the pathogenicity of *B. pseudomallei* and *B. mallei* is derived from their inability to assimilate arabinose (83).

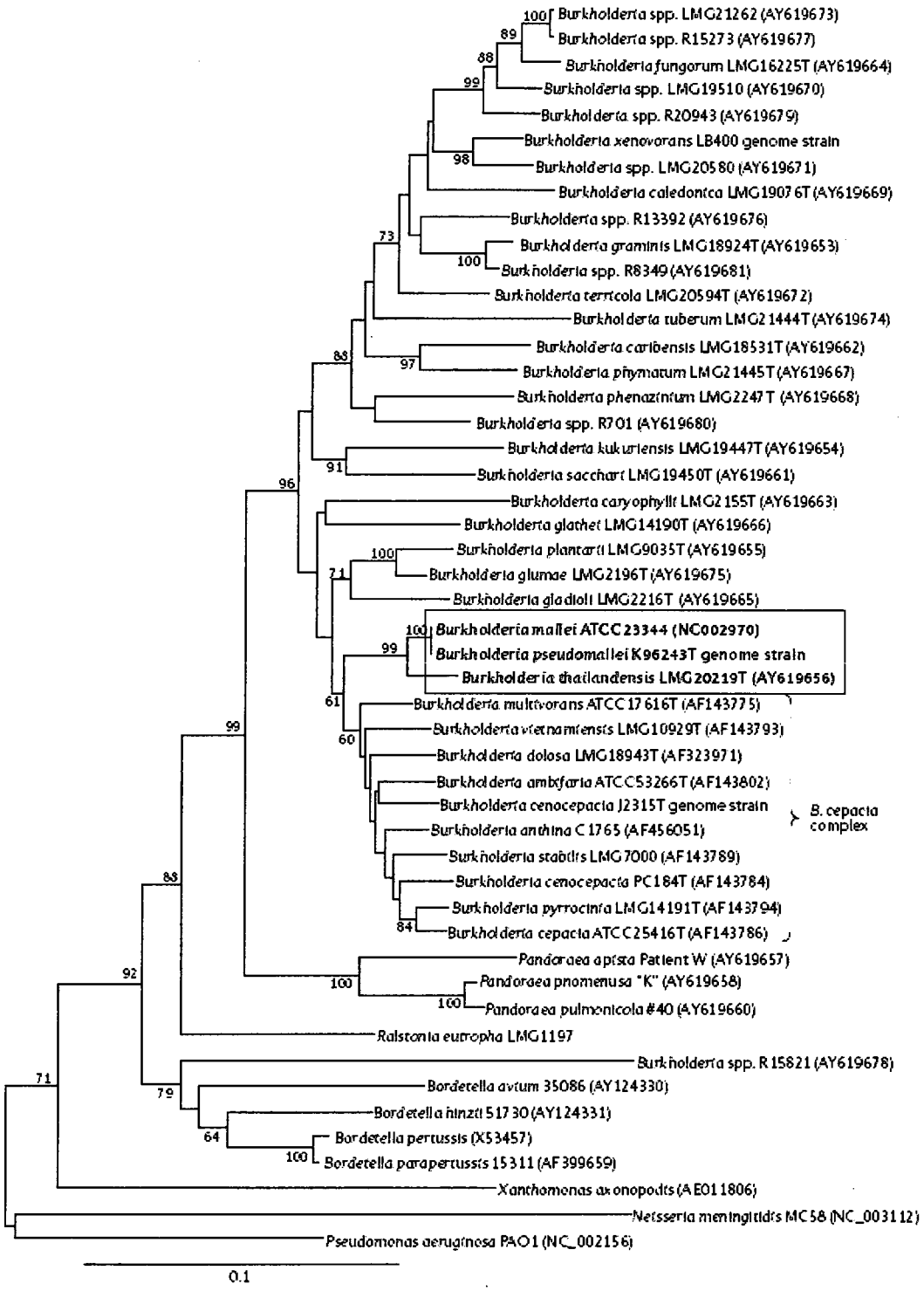

Figure 1-1 Phylogenetic tree based on *recA* sequences for *Burkholderia* species and closely related organisms. *B. mallei, B. pseuduomallei*, and *B. thailandensis*, which are highlighted by the red box, are all very closely related This figure was taken from (93) where the investigators used the Jukes and Cantor matrix model to construct the tree.

A microarray study by Ong et al. demonstrated that there was significant gene deletion in both *B. thailandensis* and *B. mallei* as compared to *B. pseudomallei* and that most of the deletions (70%) occurred on the smaller chromosome, which encodes mostly accessory functions of *B. pseudomallei* (91). Deletions in the smaller chromosome most likely reflect differences in survival mechanisms for the specific ecological niches of each of these three species.

1.4 Melioidosis

B. pseudomallei is the etiologic agent of melioidosis. The term melioidosis was introduced in 1925 by Stanton and Fletcher and comes from the Greek prefix "melis," meaning distemper of asses, and the suffix "eidos," meaning resemblance (15, 71, 138). Melioidosis is also known as Whitmore's disease, after the pathologist who first described *B. pseudomallei*, Alfred Whitmore (15). During the Vietnam War, melioidosis came to be known as "helicopter's disease" and the "Vietnamese time bomb" because there was a high rate of latent (up to 26 years post exposure (78)) infections in troops working around helicopters in Vietnam (42). First recognized in Burma in 1911, melioidosis is now known to be endemic to most of southeast Asia as well as northern Australia (28). Melioidosis can be separated into five categories: disseminated septicemic, non-disseminated septicemic, localized, transient bacteremic, and unconfirmed infection (87). The clinical states of these five forms will be discussed in section 1.4.5.

Melioidosis can occur in a variety of animals including but not limited to humans, sheep, goats, swine, monkey, gibbon, orangutan, kangaroo, wallaby, deer, buffalo, cow, camel, llama, zebra, dog, cat, horse, mule, parrot, rat, hamster, rabbit, guinea pig, ground

squirrels, seal, dolphin and crocodile (112). Although *B. pseudomallei* is a common soil isolate in areas to which it is endemic, it only rarely causes serious disease and is therefore classified as an opportunistic pathogen (42). Even as an opportunistic pathogen, it is interesting to note that the lethal dose ranges from an LD_{50} of less than 10 colony forming units (cfu) in Syrian hamsters to an LD_{50} of 10^2 cfu in mice, both of which are relatively low (although, this is largely dependent on the route of infection, animal species, and bacterial strain); no data exist on the LD_{50} for humans (37).

1.4.1 Epidemiology

Known to be endemic in southeast Asia and northern Australia, melioidosis is a tropical disease occurring in most places between the latitudes of 20° N and 20° S. Cases occurring in non-tropical locations such as Europe and the United States have been 'imported' by travelers or individuals who moved to these areas from an endemic region (33). The worldwide distribution of melioidosis is illustrated in **Figure 1-2.**

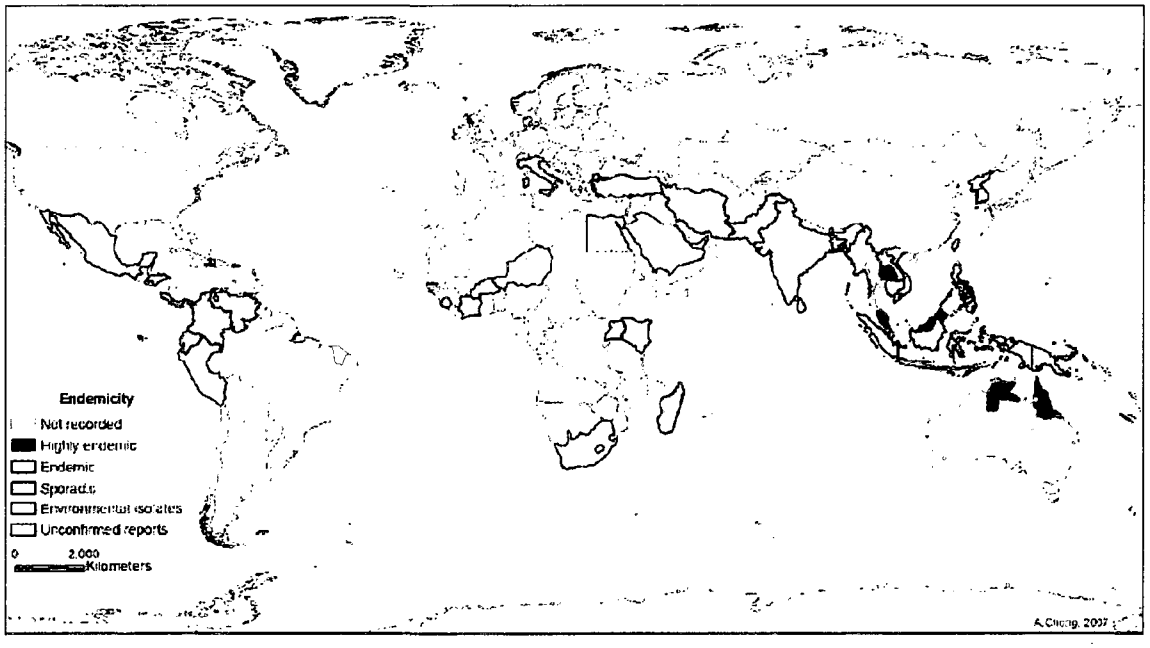

Figure 1-2 Map of global distribution of *B. pseudomallei* and melioidosis. This figure was taken from (25).

It is likely that melioidosis exists in parts of the world not highlighted on this map; due to lack of physician awareness and unavailability of microbiological facilities, the incidence of disease is probably underreported in many areas. The most complete epidemiologic information on melioidosis comes from southeast Asia and Australia, both of which are hyperendemic for melioidosis.

The epidemiology of melioidosis differs slightly between southeast Asia and Australia. For instance, while the bacteremia rate for the two regions is similar, in Thailand there is a 44% mortality rate associated with bacteremia, while in Australia the mortality rate is significantly lower, 19% (28). One explanation for this incongruence is, in Australia, rural patients may be transferred to urban areas where more advanced clinical care is available. In contrast, in Thailand, acute rural patients may be treated in community hospitals with a lower standard of care. In Ubon Ratchathani, Thailand, the average annual incidence rate of melioidosis is 4.4/100 000 (1987-1991), whereas in the Top End of the Northern Territory, Australia, the rate is much higher at 16.5/100 000 (1989-1998) (29, 121). The factors contributing to the difference in incidence are unclear, although it is possible that the lower incidence in Thailand is due to under-diagnosis during this period resulting from a lack of microbiological infrastructure (15).

Seroprevalence, as measured by indirect haemagglutination (IHA), can be up to 80% in Thailand while in Australia seroprevalence is between 5-13% (28, 62). A recent study indicated that antibodies from patients with melioidosis do not recognize *B. thailandenis* antigens (126). Therefore, environmental exposure to *B. thailandensis*, an organism found in Thailand but not Australia, does not account for the difference in seroprevalence between these two regions. Further research will be required to determine

why seropositivity is higher in Thailand compared to other hyperendemic regions (ie northern Australia) as well as to elucidate any links between seroprevalence and incidence rates. A study in Thailand demonstrated that there was an uneven distribution of *B. pseudomallei* in the soil throughout Thailand and that the regions with the highest soil isolation rates also had the highest incidence of disease (135). Perhaps there is also a link between seropositivity and magnitude of environmental exposure.

Risk factors for melioidosis are constant independent of geographical differences and include male gender, type II diabetes mellitus (37-60%), thalassaemia (44%), alcohol abuse (39%), chronic lung disease (27%), and chronic renal disease (10%) (15, 121). Rice farming as an occupation in Thailand and Aboriginal ethnicity in Australia are also risk factors (15). Surprisingly, HIV is not a risk factor for *B. pseudomallei* infection (19).

1.4.2 Transmission

During the Vietnam War melioidosis was dubbed 'helicopters disease' because many of the infected soldiers worked in helicopters. The wind disturbance caused by the helicopter was hypothesized to aerosolize *B. pseudomallei* present in the soil, at which point the soldiers would inhale the bacterium and become infected. Besides inhalation, the two other presumed routes of infection for *B. pseudomallei* are inoculation and ingestion (15).

Melioidosis is a seasonal disease; the highest incidence occurs during the rainy season in endemic regions (30). During the rainy season, pneumonic melioidosis increases and infections are more severe compared to infections occurring in other seasons (30). It is unclear whether increased precipitation acts as an environmental cue for *B. pseudomallei* to switch from a dormant (possibly VBNC) state to a more infectious

state or the inclement weather simply facilitates aerosolization of the bacterium from the soil allowing for easier dissemination via inhalation (58).

Percutaneous inoculation following exposure to muddy water in endemic areas is an important route of infection, especially in northeastern Thailand where rice farming is the most prevalent occupation (24, 71). However, the recent observation that foci of infection may include both rice field workers and individuals with limited exposure to contaminated soil is shifting opinion from a percutaneous inoculation model of infection to ingestion as a primary mode of transmission [Sharon Peacock, International Workshop on Melioidosis and Glanders Animal Models, Baltimore, MD, February 26, 2009]. This notion is supported by several well-documented outbreaks caused by contaminated water sources which were controlled following chlorination of the water (31, 55). Ingestion as a mode of transmission has important implications in the use of *B. pseudomallei* as a bioweapon, making this an important area of research in the United States where this organism is listed by the Centers for Disease Control and Prevention (CDC) as a category B select agent.

Some unusual but documented routes of transmission include aspiration following near drowning (20, 70, 92, 98), zoonotic transmission (21, 99), nosocomial infection (5), vertical transmission from mother to child (1), and sexual transmission (79). A high rate of genitourinary infection in Australia may be indicative of sexual transmission, however, this presumption requires further investigation (28).

1.4.3 Pathogenesis

Burkholderia pseudomallei evades the host immune response in part by resistance to complement, lysosomal defensins, and cationic peptides (138). Furthermore, it's

production of proteases, lipase, lecithinase, catalase, peroxidase, superoxide dismutase, haemolysins, a cytotoxic exolipid, and multiple siderophores contribute to this organisms pathogenesis but will be discussed in section 1.4.4 virulence factors (138).

In a SWISS mouse model developed to study the pathology of melioidosis following intraperitoneal inoculation, researchers noted a marked organ tropism of *B. pseudomallei* for the spleen and the liver, with the spleen having the highest bacterial load of all organs followed closely by the liver and, in far lesser amounts, the lungs, kidney, and bone marrow (45). The heart and brain display the lowest bacterial loads (45). This is slightly different in humans where lung, spleen, kidney, and prostatic abscess occur most often; but, similar to the mouse model, brain and heart involvement occurs infrequently (28). Additionally, the SWISS mouse model showed that focal growth (i.e. severe splenic abscess) can occur without overt signs of infection; this may explain the rapid death associated with acute melioidosis (45).

Burkholderia pseudomallei is a facultative intracellular pathogen that can survive in macrophages, neutrophils and monocytes (40, 60, 139). It was suggested that *B. pseudomallei,* which has the ability to survive in acidic environments, may reside in phagolysosomes as well as other membrane-bound compartments (139). Electron microscopy of the SWISS mouse spleen revealed that *B. pseudomallei* was not only densely packed into vacuoles but was also replicating inside them (45). *Burkholderia pseudomallei* escapes from membrane bound compartments by lysing the membrane (139). This is followed by actin polymerization at one bacterial pole, which induces host cell membrane protrusion into neighboring cells, allowing cell-to-cell spread of the bacteria (63).

1.4.4 Virulence

Burkholderia pseudomallei is an environmental organism and an opportunistic pathogen. Therefore, many of the characteristics identified as virulence factors may be so incidentally; their primary function being competition or survival in the organism's natural niche. Nevertheless, **Table 1-2** presents a list of virulence factors, their associated genes, and the function in virulence (if known).

Table 1-2 Defined and putative virulence factors associated with *B. pseudomallei*.

Virulence factor	Gene(s)	Function	References
actin polymeriztion	*bimA*	intracellular spread	(114)
TTSS3	*bsa* operon	escape from phagolysosome and intracellular spread	(117, 136)
	bopE	induction of actin rearrangement	(115)
	bopA	lysis of cellular membranes	(116)
	bsaZ	secretion machinery	(116)
	bipB	translocation	(120)
	bipD	translocation	(116, 134)
type I pilus	*fimA, fimC, fimD*		(15)
type IV pilus	*pilA*	adherence to epithelial cells	(41)
capsule	*wab* operon	immune evasion, attachment to epithelial cells, complement resistance	(15, 102, 103, 113)
flagella	*fliC*	macrophage and non-phagocytic cell invasion	(22, 23)
catalase	*katC*	resistance to reactive oxygen species	(74)
serine matalloprotease	*mprA*		(69, 132)
quorum sensing	*pmlI, pmlR*	repression of *mprA*	(132)
quorum sensing	*bpsI, bpsR*	negative effect on siderophore secretion, positive effect on PLC secretion	(64, 110)
RND efflux	*bpeAB-oprB*	C8HSL efflux	(9)
RND efflux	*amrAB-oprA*		(82)
TTSS1	*sctC-W*$_{BpTTSS1}$		(15, 136)
TTSS2	*sctC-W*$_{BpTTSS2}$	exoproduct secretion	(35, 100, 123, 139, 140)
siderophore	*mba, mbaF, fmtA, mbaJ, mbaI*	iron acquisition	(2)
two component system kinase	*BPSL2025*	interruption of host cell signaling	(129)
phospholipase C	*plc-1, plc-2*	cleavage of phospholipids, interruption of host cell signaling	(65)
tyrosine phosphatase	*acpA*	signal transduction	(15)

In addition to the virulence factors described above, there has been speculation about production of an endotoxin by *B. pseudomallei,* especially in those strains causing neurological forms of melioidosis. There is some controversy as to whether neurological forms of melioidosis are caused by direct invasion of the neural tissue by *B. pseudomallei* or by an endotoxin mediated process. Using *Caenorhabditis elegans* as a model, O'Quinn et al. found that *B. pseudomallei* produced an endotoxin that killed the nematode by interfering with the nematode's ability to restore Ca^{2+} membrane potentials leading to paralysis and ultimately death (90). The translation of these findings to vertebrate models is questionable, especially since *B. thailandensis* was more pathogenic than *B. pseudomallei* in this model, which is exactly opposite of the relative infectivity observed in humans and other vertebrates. Another study in the same time frame by Gan et al. found that *B. mallei* and *B. pseudomallei* were more pathogenic to *C. elegans* than *B. thailandensis*; they attribute the contrast in their findings and the O'Quinn findings to strain and media differences (44). In either case, use of an inexpensive and easily manipulated surrogate host is an interesting approach to defining virulence factors and may have high-throughput capacity not feasible in a mouse model. In fact, another group is using a wax moth model to define the 'virulome' of *B. pseudomallei*; so far, their results indicate that mutant strains with reduced virulence in mouse models also have reduced virulence in the moth, however, the reciprocal is yet to be seen (106).

In comparison to other Gram negative organisms, there is a deficit of knowledge regarding *B. pseudomallei* virulence determinants. This can be contributed to two factors; first, *B. pseudomallei* was generally not a focus of basic scientific research until its listing as a category B select agent in 2001. Second, a lack of genetic tools which

were both select agent compliant and functional in *B. pseudomallei* hindered initial research efforts. Recent development of such tools including Tn*5*-based transposition system (36), Himar1-based transposition system (104), and several systems for allelic exchange (6, 47, 125) will accelerate efforts to identify and verify important *B. pseudomallei* virulence factors.

1.4.5 Clinical State

The most common symptoms of melioidosis are fever and respiratory symptoms which include cough, dyspnoea, and chest pain (52). Other symptoms include abdominal pain, vomiting, diarrhea, dysuria, and hematuria (52). Associated with 50% of all cases, pneumonia is the most common clinical syndrome associated with *B. pseudomallei* infection. Lung involvement following percutaneous inoculation indicates that pneumonia occurs secondary to sepsis; this is supported by a null correlation between radiographic evidence and patients' severe clinical status (15). Besides pneumonia, *B. pseudomallei* infections can lead to a variety of clinical manifestations, including abscesses in nearly every organ. One study demonstrated clonality between strains that caused neurological symptoms and strains from pneumonic melioidosis, indicating host factors or routes of transmission may play an underappreciated role in the clinical manifestations of *B. pseudomallei* infections (31). Route of infection, strain-specific virulence determinants, and host factors all contribute to the clinical picture of each melioidosis case.

There are a few geographically distinct differences in clinical presentation of melioidosis. In southeast Asia, acute suppurative parotitis occurs in up to one third of pediatric cases of melioidosis (138). These children have fever, pain, and swelling over

the parotid gland and delayed treatment can lead to permanent Bell's Palsy (138). Suppurative parotitis has only been documented once in Australia; although it is important to note that the overall number of pediatric cases of melioidosis in Australia is relatively low (15, 75). In Australia 4% of melioidosis patients present with encephalomyelitis and 18% of males with melioidosis have prostatic abscess; these presentations are almost exclusive to northern Australia (27, 29). High incidence of prostatic abscess in Australia mandates imaging for detection followed by drainage, as this type of abscess does not respond well to antibiotic treatment alone.

Melioidosis can have either an acute, sub-acute, chronic, or sub-clinical (latent) course of disease. Acute and sub-acute disease presentations are similar (fever, visceral abscess, pulmonary distress, general malaise) and differ primarily in that acute melioidosis, if untreated, can lead to rapid death while untreated sub-acute melioidosis can take weeks to months to cause death. Chronic melioidosis is characterized by illness lasting longer than two months. Sub-clinical infection may go undetected until activated by some unknown event (perhaps by trauma). Cases of sub-clinical melioidosis have gone 26-62 years from initial inoculation to manifestation of clinical disease (activation) (78, 86).

Even with treatment, mortality associated with any of the 3 disease presentations remains at 20-50% (15). Despite adequate treatment, relapse occurs in up to 23% of cases (26). Seventy-five percent of recurrent melioidosis is due to relapse (reactivation of original infection), while the remaining 25% are due to re-infection with a new strain (76). However, a more recent study by Pitt et al., which used BamHI ribotyping to analyze multiple isolates from single patients during an initial infection, states that 27%

of patients are initially infected with multiple strains (the initial relapse vs. re-infection study did not account for this possibility) indicating that the 25% of cases due to re-infection could actually be relapse of a strain not identified in the original infection (97). It is important to note that initial infection with multiple strains as a common occurrence is a point of contention, as a recent study by Limmathurotsakul et al. contradicts the findings of Pitt et al. (72, 97).

1.4.6 Diagnosis

Based on clinical features alone, melioidosis cannot be differentiated from infections caused by other organisms. Early and specific laboratory diagnosis is crucial because *B. pseudomallei* is highly resistant to many antibiotics and, if left untreated, melioidosis can have mortality rates as high as 80%. Currently, culture of *B. pseudomallei* from a clinical specimen is the only reliable method of diagnosis for melioidosis (94). Due to the lack of microbiological facilities in some areas where melioidosis is endemic as well as the lack of skilled personnel in non-endemic areas, alternative diagnostic tests are a necessity for both naturally occurring melioidosis and intentional release of the etiologic agent. Serology, antigen detection, and PCR have been evaluated for diagnostic use, however, there are conflicting reports on the applicability of these diagnostic techniques (46, 59, 77, 89).

To identify *B. pseudomallei* by culture, the clinical specimen must be cultured and sub-cultured on selective media followed by biochemical characterization (109). Clinical specimens to be tested should include blood, urine, throat swab and respiratory secretions (94). Colonization of healthy individuals with *B. pseudomallei* has not been observed therefore, culture of the organism from even a non-sterile site (such as the throat) is

indicative of melioidosis (18). The selective medium of choice is Ashdown's selective agar which is trypticase soy agar supplemented with 4% glycerol, 5 mg/L crystal violet, 50mg/L neutral red, and 4 mg/L gentamicin (modified Ashdown's also contains colistin) (4, 143). A few of the drawbacks of the culture method of diagnosis are that it requires skilled personnel for interpretation of the results, 3-4 days are required for definitive diagnosis, and the normal flora from a clinical specimen can overgrow *B. pseudomallei* because of its relatively lengthy generation time (109). Additionally, Ashdown's is not commercially available in non-endemic areas which has important implications for diagnosis following intentional release of *B. pseudomallei* (94). A study comparing several commercially available alternatives to Ashdown's found that *B. cepacia* media was sensitive and specific enough to be used as an alternative in non-endemic areas (94, 95). Some recent advances in the culture method include the BacT/Alert® nonradiomatric blood culture system, which can detect 90% of positive *B. pseudomallei* infections within 48 hours, and the API 20NE test panel, which simplifies identification (109). The reported sensitivity of the API 20NE test panel ranges wildly from ~37-99% rendering its clinical use questionable (3, 34, 56). Both of these methods are costly and therefore their applicability in endemic areas is questionable.

Antibody detection in endemic areas for the diagnosis of melioidosis has been evaluated by several groups (38, 89, 107, 109, 141). The primary challenge with serological diagnostics for *B. pseudomallei* in endemic regions is the presence of antibodies, either cross-reacting or produced during subclinical infections of healthy individuals, leading to a false positive diagnosis (77). Typically, serologic diagnostic tools, such as the indirect hemagglutination assay, have much lower sensitivity in the

Thai population as compared to the Australian population. As discussed earlier, the reasons for this are not clear but it is not due to cross-reactivity due to exposure to *B. thailandensis.* The effort to identify novel *B. pseudomallei* antigens is ongoing (50, 124) and will facilitate improvement of antibody detection for diagnosis of melioidosis.

Antigen detection in serum samples is a superior method of diagnosis because it indicates active disease, thus circumventing the issue of high levels of seropositivity in endemic areas (13, 109). A monoclonal antibody assay for the detection of exotoxin, an ELISA for the detection of a novel 40-kDa secreted protein, an ELISA for the detection of LPS in unconcentrated urine, an ELISA for the detection of the 200-kDa secreted protein, latex agglutination, and immunofluorescence tests have all been developed for the diagnosis of melioidosis (73, 109). Of these tests, the latex agglutination test made with monocolonal antibodies specific to the 200-kD exopolysaccharide of *B. pseudomallei* demonstrates the most promise as a diagnostic tool, with a sensitivity of 99.5% and a specificity of 100% (3).

Several molecular methods involving PCR have been evaluated for their use as *B. pseudomallei* diagnostics (43, 61, 88, 127, 130). Unfortunately, the lower limit of detection for most of these tools is above that of most clinical specimens making their practical application questionable (94). Although, a report by Inglis et al., illustrates the usefulness of molecular methods as supplementary diagnostic tools while another report by, Ulrich et al., highlights the use of PCR for differentiation of *B. pseudomallei* and *B. mallei*, both of which would be useful in the event of intentional release of either organism (57, 131).

In a large-scale, double-blind, case-controlled study by Sirisinha et al. carried out in a real clinical setting, diagnosis via detection of antibodies, antigen, and genetic material of the same clinical specimens was evaluated (109). The study concluded that the method of choice remains the culture on Ashdown's agar, not only because of its reliability but also because of its cost effectiveness and simplicity (109). The study also suggests that in order to reduce the time needed for diagnosis the culture method could be supplemented with one of the available serological or molecular methods (109).

1.4.7 Prevention and Prophylaxis

Preventative measures against naturally occurring melioidosis include covering all open wounds with waterproof dressings and wearing boots and gloves when in contact with water-logged soil in tropical locations, especially for individuals with known risk factors (52). Preventative measures for laboratory exposure to *B. pseudomallei* include conducting all research involving live culture in a biosafety level three laboratory (BSL3) and adhering to all guidelines outlined in the Biosafety in Microbiological and Biomedical Research Laboratories manual (96). Despite ongoing efforts to develop a vaccine for melioidosis, none are available to date (8, 39, 48, 51, 85, 139).

Trimethoprim-sulfamethoxazole, amoxicillin–clavulanic acid, doxycycline, and fluoroquinolones have all been evaluated in mouse models for their use as post-exposure prophylactics (96, 118). In order to have any efficacy, all tested prophylactic regimens had to be administered within the first 48 hours of exposure (96). Trimethoprim-sulfamethoxazole (co-trimoxazole) has the most promising prophylactic effects and is currently recommended as prophylaxis in high risk laboratory exposures (96). Fluoroquinolones are the only drug tested that had no prophylactic effects and their use is

not recommended (118). In the event of laboratory exposure Peacock et al., recommend co-trimoxazole taken every 12 hours for a course of three weeks (96). In the event of intentional release, the United Kingdom recommends a seven day course of either doxycycline or co-trimoxazole taken twice daily (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/119494741244). The United States recommends administration of co-trimoxazole in response to the same events (118).

Even if prophylactic treatment is not recommended (whether in laboratory exposure or in response to deliberate release), all potentially exposed persons should self-monitor their temperature for up to 21 days. If a temperature >38° C is observed medical attention should be sought immediately (96). Additionally, because onset of disease can be delayed up to 62 years post-exposure, any potentially exposed person should notify healthcare personnel of their exposure should they develop febrile illness at any point in their lifetime (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/119494741244).

1.4.8 Treatment

Thirty years ago the conventional treatment for melioidosis consisted of a quadruple-drug regimen of intravenous chloramphenicol, doxycycline, and co-trimoxazole (66, 111). However, with a mortality rate of 50-90%, this was not an effective treatment, mostly due to the bacteriostatic nature of these drugs rendering them ineffective in the management of acute melioidosis (111). In 1989, a version of the current treatment, which consists of two phases, was employed. These two phases are described as an initial intensive phase followed by a long course of maintenance or eradication therapy (15). The initial intensive phase consists of high dose intravenous

ceftazidime, imipenem, or meropenem for a duration of 10-14 days followed by 12-20 weeks of maintenance therapy with either a combination of oral co-trimoxazole and doxycycline (Thailand), or co-trimoxazole monotherapy (Australia) (15, 138). The addition of ceftazidime to the treatment regimen has dramatically improved thearpy, reducing mortality up to 60% over the original four drug regimen (111).

Maintenance therapy is required because *B. pseudomallei* can escape the immune system as well as antibiotic killing due to its intracellular nature. However, maintenance therapy also poses several problems for the treatment of melioidosis. First, treatment is extremely expensive, costing up to $100 per day; for a course of treatment lasting twenty weeks it would cost upwards of $14,000 for maintenance therapy alone (15). Secondly, due to both the length and expense of treatment as well as adverse side-effects of the antibiotics, compliance to the antibiotic regimen is low (15, 71).

In endemic regions, treatment of melioidosis is associated with a 50% compliance rate and non-compliance is apparently the most important factor in determining relapse (15). Relapse often necessitates a change in the course of therapy which is problematic due to the high intrinsic resistance of *B. pseudomallei* to most antibiotics (122). Besides third generation cephalosporins (ceftazidime), the only other drugs proven to be effective for the intensive phase of treatment are the carbapenems (meropenem and imipenem) (15, 16, 80). In the event of resistance to co-trimoxazole; doxycycline, chloramphenicol or co-amoxiclav could be used for maintenance therapy, however, these drugs are also associated with a high rate of relapse (11, 14, 17, 101).

There are several potential problems with the current melioidosis treatment regimen, relative to both treatments in endemic areas and in response to a deliberate

release of *B. pseudomallei,* which need to be addressed. All of the drugs currently available for the initial intensive phase have to be given intravenously which necessitates hospitalization and increases the cost of treatment. Up to 13% of all clinical isolates are resistant to co-trimoxazole which is the only reliable drug for maintenance therapy (142). Prolonged intensive therapy, without maintenance treatment, may be sufficient treatment for melioidosis (and a way to circumvent co-trimoxazole resistance), as it is an effective treatment for other Gram negative infections; however, clinical evaluation is required to validate this suggestion with regard to melioidosis (15). Emergence of resistance to the already limited treatment options for melioidosis is imminent; therefore, it is prudent to understand the underlying mechanisms of such resistance in order to develop more effective treatment strategies (12, 128, 133).

1.5 *Burkholderia pseudomallei* as a biological weapon

Burkholderia pseudomallei has recently become a focus of basic scientific research in the United States as a result of the Centers for Disease Control's (CDC) recognition of the potential for this organism to be used as a bioweapon. There are five basic characteristics that give an organism the potential to be used in biological warfare: the organism should be easily attainable and able to be grown to high titers, exhibit high virulence leading to high morbidity/mortality, be easily disseminated, be stable in harsh environments, and enable selective targeting (i.e. those responsible for dissemination can be protected) (68). *B. pseudomallei* has been listed by the CDC as a category B potential biological agent. Category B is the second highest priority classification and requires that an organism be moderately easy to disseminate, cause moderate morbidity but low mortality, and requires enhancement of CDC's diagnostic abilities as well as enhanced

disease surveillance. Classification of *B. pseudomallei* as a category B agent indicates that it meets, at least partially, the criteria described above which constitutes potential for use as a biological weapon.

Specifically, *B. pseudomallei* is readily attainable, as it can be easily cultured (using simple medium) from soil of endemic regions, including southeast Asia and northern Australia (32). Virulent *B. pseudomallei* requires no special growth conditions and a culture will usually grow to saturation in 24 hours at 37° C. Virulence and morbidity and mortality of *B. pseudomallei* when used as a bioweapon can be estimated from morbidity and mortality rates in endemic areas. Incidence of *B. pseudomallei* in endemic areas ranges from 16.5-41.7/100,000 with associated bacteremia rates ranging from 46%-60% (depending on geographical location) and mortality rates of 19%-44% (15, 28). This indicates that *B. pseudomallei* causes moderate morbidity and low to moderate mortality, both of which could potentially be enhanced by genetic engineering. Dissemination of *B. pseudomallei* would be fairly easy as transmission through water supply as well as inhalation have been documented, although, as described in section 1.4.2, these are not the major routes of transmission in endemic areas (29, 31). Person-to-person transmission seems to be rare, thus dissemination would be limited to people who come into contact with the initial contaminant (37). The natural environment for *B. pseudomallei* is soil; therefore, it is reasonable to assume that it would be able to survive relatively harsh conditions despite its inability to form spores. Selective targeting can be achieved through the route of dissemination used by the perpetrator; for example, if contamination of the water supply was chosen, the perpetrator could avoid use of contaminated water. The potential for use of *B. mallei,* also a category B select agent,

was realized during World War I when this organism was intentionally released by the Central Powers to immobilize the Russian horses (15).

As a direct result of the status of *B. pseudomallei* as a select agent, many restrictions have been implemented in regard to the manipulation of this organism. First, all research entities and investigators working with the organism must be registered and pass a FBI background check. Transfer of the organism between research groups is tightly regulated by both the CDC and the United States Department of Agriculture (USDA). The organism can only be manipulated under BSL3 conditions by specially trained personnel using appropriate personal protective equipment. Finally all tools for genetic modification of *B. pseudomallei* must fulfill specific requirements, such as use of only approved antibiotic selective markers. These restrictions make working with this organism expensive both in terms of both equipment and time; therefore, many investigators are interested in the development and utilization of surrogate model organisms in order to facilitate research that is difficult to conduct under these conditions (49, 67, 81, 137). A better alternative to surrogate bacteria would, of course, be availability of an exempt avirulent *B. pseudomallei* strain that could be handled under BSL2 conditions.

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CHAPTER 2

ANTIBIOTIC RESISTANCE

2.1 General information on antibiotic resistance

Antibiotic resistance is a threat to the treatment of most bacterial infections including melioidosis. As discussed earlier, the treatment of melioidosis is difficult because there are a limited number of treatment options due to the high intrinsic antibiotic resistance of *B. pseudomallei*. In order to develop new treatments to circumvent antibiotic resistance, it is necessary to understand the underlying mechanisms of resistance. Clinically, susceptibility of an organism to a drug is numerically determined using the minimum inhibitory concentration (MIC) as a guideline (63). In order for a drug to be considered an acceptable treatment, concentration of the drug in the serum must reach or exceed the MIC (25). Given that the MIC can be accomplished in serum levels, there are two possible scenarios for antibiotic resistance: the organism never displays sensitivity to a drug (initially has a very high MIC) or the organism initially displays sensitivity followed by increasing insensitivity (observed increase in MIC over time) (76). Thus, antibiotic resistance can be classified into two broad categories: intrinsic, which is described by the former scenario, and acquired, as in the latter scenario.

Intrinsic resistance is low susceptibility of a bacterium to an antibiotic as a function of the natural properties of the bacteria (3). Examples of intrinsic resistance

include the impermeability of the Gram negative outer membrane and the presence of constitutively expressed efflux pumps. Intrinsic resistance factors are chromosomally encoded, and are important because they automatically limit the treatment options for many infections (25, 27, 76).

Acquired resistance occurs when a resistant strain emerges from a previously susceptible bacterial population (76). The emergence of a resistant strain is largely due to mutations in chromosomal genes or introduction of foreign resistance elements via mobile genetic elements (i.e. integrons, plasmids, and transposons). Mutations leading to drug resistance occur at the same frequency as other mutations (10^{-6} to 10^{-10}), and it has been proven that, generally, antibiotics do not induce mutation, but rather act as a screening agent to select for previously occurring mutations (21, 43, 52, 102). The use of antibiotics for 'selection' of resistant mutants has been exploited by researchers to elucidate mechanisms of resistance to many antibiotics (11, 18, 24, 64, 71, 79).

Resistance is an immediate problem because it can lead to elimination of previously valuable treatments, forcing the use of alternative treatments that can be more expensive or cause worse side-effects (87). For example, in the case of ceftazidime resistant *B. pseudomallei*, co-amoxiclav is sometimes substituted for treatment. However, co-amoxiclav is associated with a higher treatment failure rate as well as a higher rate of relapse; there are few comparable substitute for ceftazidime as treatment for melioidosis (75, 85) with the exception of carbapenems such as meropenem. Resistance is of particular concern in infections that require long courses of antibiotic treatment, such as *M. tuberculosis* and *B. pseudomallei*, because prolonged exposure to antibiotics selects for resistant bacteria, leading to failure of treatment (35, 90, 98, 103). Exposure to sub-

inhibitory concentrations of antibiotics is also particularly problematic. *In vivo* and *in vitro* exposure of *B. pseudomallei* to sub-inhibitory concentrations of ceftazidime and quinolones results in both filamentous cells and small colony variants (SCV) which are resistant to ceftazidime as well as several other structurally unrelated antibiotics (16, 30). Neither the mechanisms for resistance nor the changes in cell physiology have been elucidated in SCV or filamentous cells; however, both could have implications in the management of melioidosis and frequency of relapse.

2.2 Mechanisms of antimicrobial resistance in bacteria

Both intrinsic and acquired resistance is a result of three main mechanisms for resistance: (i) alteration of the antibiotic target, (ii) enzymatic drug inactivation, and (iii) decreased antibiotic accumulation. These three mechanisms can act individually or simultaneously to have a synergistic effect on resistance (86). All three of these mechanisms have not yet been described in *B. pseudomallei*. However, this organisms' resistance to nearly every class of antibiotics is indicative of the involvement of all three mechanisms, the details of which are discussed in the following sections.

2.2.1 Alteration of antibiotic target

Generally speaking, resistance due to alteration of the antibiotic target is a result of accumulation of chromosomal mutations (102). Cells containing these chromosomal mutations are selected during treatment with an antibiotic that acts at the non-mutated target resulting in a population switch from susceptible to resistant. Altering the target of the antibiotic can include modification such that it is insensitive to the antibiotic (yet still capable of normal physiological function), duplication of the target (with the duplicate

being resistant to the antibiotic), or reduction of the physiological importance of the target (25, 76, 102).

Resistance to quinolones as a result of antibiotic target modification has been described in *B. pseudomallei* (and many other bacteria). Quinolones target the function of topoisomerase II (*gyrA)* in Gram negative bacteria. Mutations occurring between amino acids 67 and 106, result in modification of topoisomerase II, such that it is still functional but is no longer susceptible to inhibition by quinolones; thus this region has been dubbed the quinolone resistance determining region (QRDR) (12). Victorov and colleagues found point mutations in *gyrA* resulting in amino acid transitions at positions 83, 81, and 87 in fluoroquinolone resistant mutants of *B. pseudomallei* (94).

Resistance to trimethoprim and sulfamethoxazole has been documented in *B. pseudomallei* (83, 87, 97). Trimethoprim is a known substrate for efflux in *B. pseudomallei* however, work described in Chapter 4 demonstrates trimethoprim resistance in strains lacking expression of BpeEF-OprC, the pump for which trimethoprim is a substrate. One possible mechanism for this resistance is mutations in the target for trimethoprim, dihydrofolate reductase (DHFR). No mechanism for sulfonamide resistance in *B. pseudomallei* has been documented in the literature to date. However, mutations in the sulfonamide target enzyme, dihydropteroate synthase (DHPS), have been shown to convey resistance to sulfamethoxazole in other bacteria (6, 45). Our lab is currently investigating whether the DHFR and DHPS genes, *folA* and *folP*, from co-trimoxazole resistant strains lacking efflux expression contain mutations which are responsible for observed resistance. Understanding possible mechanisms for co-

trimoxazole resistance is essential because the current treatment regimen for melioidosis includes at least 20 weeks of co-trimoxazole therapy for eradication of *B. pseudomallei.*

2.2.2 Enzymatic drug inactivation

The biochemical structure of an antibiotic is integral to its function and the efficacy of a drug can be altered by simple alterations in biochemical structure. For example, many beta-lactams differ in structure by only one or two substitutions to the lactam ring; however, these substitutions dramatically change the affinity for their binding sites (PBP), such that, although they have very similar structures, some bind well to a certain PBP, while others will not. Some bacteria have enzymes that can act deleteriously on an antibiotic, altering its structure so it is no longer effective in treatment against that bacteria (10, 25). These enzymes include β-lactamases, acetyltransferases, phosphotransferases, nucleotidyltransferases, esterases, hydrolases, glycosylases, and monoxygenases; they confer resistance to β-lactams, aminoglycosides, macrolides, chloramphenicol, and tetracyclines (3).

Burkholderia pseudomallei chromosomally encodes at least seven β-lactamases (from Ambler classes A, B, C, and D) and one aminoglycoside acetyltrasferase (33). The aminoglycoside acetyltransferase has not been characterized, furthermore, high level aminoglycoside resistance in *B. pseudomallei* has been attributed to efflux (62). β-lactamases inactivate β-lactams by hydrolysis of the β-lactam ring; clavulanic acid is a competitive inhibitor of this reaction. β-lactamases from classes A and D have been characterized in *B. pseudomallei* and this information is summarized in **Table 2-1**. Mutations in these β-lactamases (also summarized in **Table 2-1**) indicate that there are three basic mechanisms for acquired resistance to β-lactams, with regard to β-lactamases:

derepression of β-lactamase expression, mutations in the β-lactamase leading to altered substrate profile, and β-lactamase mutations leading to β-lactamase inhibitor resistance (17, 28).

Table 2-1 Characterized β-lactamases and derived mutants from *B. pseudomallei*

β-lactamase Class	Mutant	Amino acid change	Substrate	Inhibited by clavulanic acid	Reference
Class A					
blaA$_{BPS}$ (*penA*)			Cephalosporins (not ceftazidime)	Yes	(17, 28, 48)
	BPS-1m	P167S	Ceftazidime and less activity against other cephalosporins	Yes	(17, 28, 89)
	BPS-1$_{392f}$	S72F	Cephalosporins	No	(28, 89)
Class D					
oxa57			Oxacillins	Yes	(38)
	OXA-42	K232N	Oxacillins	Yes	(38, 67)
	OXA-43	S104P	Conflicting reports of oxacillins vs. no activity	Yes	(38, 67)
	OXA-59	D170N	Oxacillins	Yes	(38)

With the exception of OXA-59, the mutant β-lactamases in **Table 2-1** were all derived from ceftazidime resistant strains which were either isolated clinically or laboratory generated. This is interesting because only one of the mutants (BPS-1m) had a significant increase in ceftazidime resistance. In fact, OXA-42 and OXA-43 were both induced in their respective ceftazidime resistant strains, but neither has any activity against ceftazidime; the authors suggest co-ordinate expression of multiple unlinked β-lactamases as an explanation but this possibility requires further investigation (67). The substrates described for the two characterized β-lactamases of *B. pseudomallei* account for the intrinsic resistance of this organism to β-lactams. However, because β-lactams are currently the only useful antibiotics against *B. pseudomallei*, further characterization of the remaining β-lactamases and their regulation would be prudent.

2.2.3 Decreased antibiotic accumulation

A certain intracellular concentration of antibiotic must be achieved in order for the drug to be effective (63). There are two ways that the accumulation of an antibiotic in relation to its bacterial target can be altered: by a change in the permeability of the cell or by active efflux of the drug from the cell (46). These are both of particular interest in the Gram negative species because they have an outer membrane which provides an extra permeability barrier and multi-drug efflux systems have been characterized for several Gram negative species (8, 15, 74, 78). Often resistance arises as synergistic cooperation of decreased outer membrane permeability accompanied by increased efflux.

Outer membrane permeability is largely controlled by the expression of outer membrane proteins that form porins through which molecules can access the cell (8). Many Gram negative species show intrinsic resistance to glycopeptide antibiotics, like vancomycin, because they are too big to diffuse through any of the porins expressed (76). Additionally, resistance to quinolones has been observed in *E. coli norB* and *norC* mutants. The gene products of both of these genes are involved in the regulation of OmpF porins (1). These mutants have altered *ompF* (the porin, OmpF, is the gene product) expression, which alters the permeability profiles of the outer membrane of these mutants, decreasing permeability and inhibiting the entry of the quinolone into the cell (1).

Although not attributed to a specific porin, inducible arsenite resistance due to decreased outer membrane permeability has been demonstrated in *B. pseudomallei* (5, 7). Analysis of the *B. pseudomallei* outer membrane revealed five major outer membrane proteins (OMPs) which were conserved among the 12 isolates analyzed (29). Of these five conserved OMPs (relative molecular masses, M_r, of 70,000, 38,000, 31,000, 24,000

and 17,000), only the M_r 38,000 protein has been characterized as a porin. This protein tetramerizes to form a porin with a diameter of 1.2-2.0 nm and a size exclusion limit, as demonstrated in **Table 2-2**, of M_r~650 (81, 82). Additionally, identical diffusion kinetics for both charged antibiotics and uncharged sugars indicate that charge has no apparent affect on diffusion through OMP-38; this is in contrast to descriptions of porins from other bacteria (81).

Table 2-2 Permeability rates of antibiotics and sugars through *B. pseudomallei* OMP-38 (adapted from (81)).[a]Relative to permeability of L-arabinose.

Sugar or Antibiotic	Mr	Permeability rate (%)[a]
L-arabinose	150	100
D-glucose	180	85
D-mannose	180	85
D-galactose	180	85
GlcNAc	221	60
D-sucrose	342	20
D-melezitose	522	8
Amikacin	782	0
Gentamicin	709	0
Ceftazidime	637	<1
Cefepime	572	4
Clindamycin	505	8
Ciprofloxacin	421	15
Meropenem	383	20

In addition to porins, the lipopolysaccharide (LPS) of Gram negative bacteria can affect the permeability of antibiotics into the cell (65). Polymixin B resistance in *B. pseudomallei* has been attributed to this organism's unique LPS structure. Burtnick and colleges suggest that the targets of polymixin B (the inner core and lipid A segments of LPS) are hidden by the outer core and O-antigen segments of the LPS such that these outer portions of the LPS create a permeability barrier between polymixin B and its interior targets (9). Finally, there is some evidence that *B. pseudomallei* forms biofilms (both *in vivo* and *in vitro*) which are more resistant to antibiotics than their planktonic

counterparts (96, 97). Although the exact mechanisms for antibiotic resistance in biofilms is unclear, one possibility is reduced permeability of antibiotics (especially through the glycocalyx which encompasses biofilms) to the interior regions of the biofilm (4, 54).

Another mechanism for resistance due to decreased antibiotic accumulation is efflux. First discovered as a plasmid-encoded tetracycline-specific resistance determinant in *Escherichia coli*, it quickly became clear that efflux was most commonly chromosomally encoded, present in both prokaryotes and eukaryotes, and could convey resistance to multiple structurally unrelated compounds simultaneously in an energy dependent manner (26, 47, 59). Drug efflux proteins can be divided into five families: ATP-binding cassette superfamily (ABC), major facilitator superfamily (MFS), multidrug and toxic compound extrusion family (MATE), small multidrug resistance family (SMR), and the resistance-nodulation-cell division family (RND) (73). Members of the MFS, SMR, MATE, and ABC families can operate as a single component while members of the RND family of transporters are always tripartite, as illustrated in **Figure 2-1.** Additionally, **Table 2-3**, summarizes important details about each family including energy source, clinically relevant substrates, and structure (in terms of number of transmembrane segments, TMS).

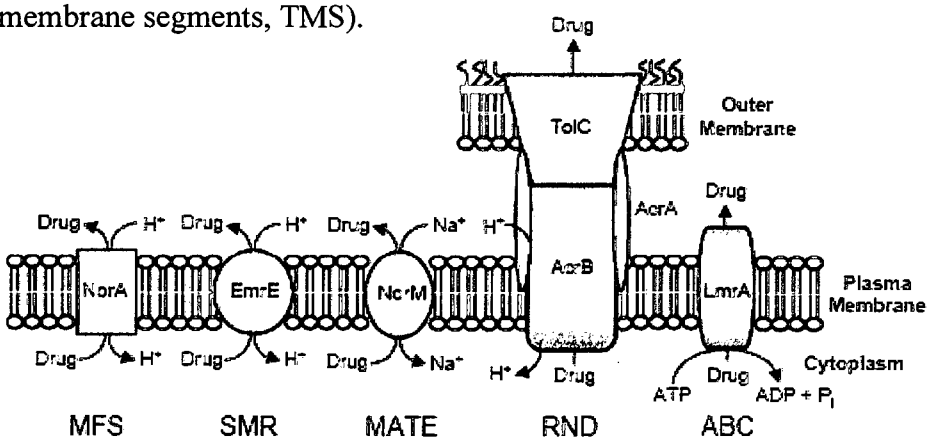

Figure 2-1 Schematic illustration of the five families of drug transporters [taken from (42)]

Table 2-3 Summary of bacterial drug transporter families. Transmembrane segments (TMS), macrolide-lincosamides-streptogramin (MLS), *Lactococcus lactis (L. lactis), Bacillus subtilis (B. subtilis), Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Serratia marcescens (S. marcescens), Vibrio parahaemolyticus (V. parahaemolyticus)*

Family	Energy Source	Structure	Substrates	Example	Reference
ATP Binding Cassette (ABC)	ATP hydrolysis	6 TMS	ethidium bromide, colchicine, MLS antibiotics, tetracycline, and chloramphenicol	*L.lactis* LmrA *B. subtilis* YheI/YheH	(73, 88, 92)
Major Facilitator Super family	proton gradient	12 TMS	fluoroquinolones, chloramphenicol, dyes, disinfectants	*S. aureus* NorA *B. subtilis* Bmr	(73, 84, 100)
Resistance/Nodulation/Cell Division (RND)	proton gradient	12 TMS	β-lactams, quinolones, macrolides, trimethoprim, aminoglycosides, tetracyclines	*E. coli* AcrAB-TolC *B. pseudomallei* AmrAB-OprA, BpeAB-OprB, BpeEF-OprC	(13, 40, 62, 66, 101)
Small Multidrug Resistance (SMR)	proton gradient	4 TMS	acriflavine, ethidium bromide, other dyes, lipophilic cations	*S. marcescens* SsmE *E.coli* EmrE	(61, 72, 77)
Multidrug and Toxic-compound Extrusion (MATE)	Na^+/H^+ drug antiport	12 TMS	aminoglycosides, fluoroquinolones, cationic dyes	*V. parahaemolyticus* NorM *S. aureus* MepA	(36, 47, 70)

As illustrated in **Figure 2.1**, RND family transporters are composed of a transporter (located in the cytoplasmic membrane), a membrane fusion protein (MFP) (which spans the periplasm), and an outer membrane protein (OMP) (which provides a conduit to the extracellular space). As a result of their unique architecture, RND transporters impart a higher degree of resistance to Gram negative bacteria compared to their single component counterparts which can only deliver their substrates to the periplasm. However, there have been reports of single component (non RND) pumps associating with MFPs and OMPs to form a conduit through the Gram negative cell envelope leading to high level resistance (42). Synergy between single component pumps and RND pumps resulting in high level tetracycline resistance has also been observed in Gram negative bacteria (44).

All three parts of the RND transporter are obligatory for functional transport, and are usually encoded on a single operon with a divergently encoded regulator, as depicted in **Figure 2-2**. Of course there are exceptions, for example, in *Pseudomonas aeruginosa*, *oprM* is only transcribed on the same operon as *mexAB*, however, it also associates with MexXY, MexJK, and MexVW (104). Additionally, not all RND gene clusters have a divergently transcribed regulator, nevertheless, expression of efflux pumps is tightly regulated by either local or global repressors/activators (42). Expression of RND efflux pumps can be constitutive, as is the case for *B. pseudomallei's amrAB-orpA* and *P. aeruginosa's mexAB-oprM*, or inducible, such as *P. aeruginosa's mexXY-oprM*. Induction of efflux pump expression is known to occur by regulator mutations, promoter mutations, quorum sensing, as well as induction by substrates of the pump (2, 13, 32, 39, 57, 60, 68, 69). In this way, RND efflux is a mechanism of both intrinsic and acquired

resistance. Some regulators of RND efflux, such as *P. aeruginosa's* MexT and *E. coli's* MarA, also modulate expression of other genes, such as those encoding porins, which ultimately functions to maximize resistance (22, 58).

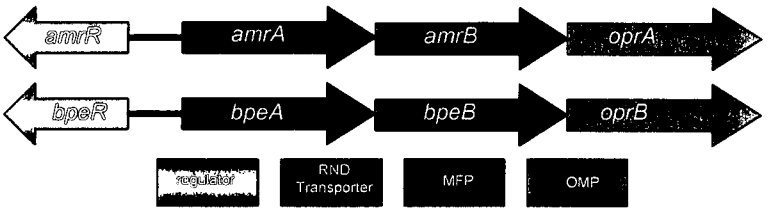

Figure 2-2 Genetic arrangement of RND pump operons *amrAB-oprA* and *bpeAB-oprB* of *B. pseudomallei*

The wide range of structurally unrelated substrates for RND pumps makes it difficult to envision a mechanism of extrusion, without which it is impossible to devise methods to circumvent this means of resistance. Recently both Lomovskaya and Nikiado have compiled all the available genetic, biochemical, and structural data about AcrAB-TolC (the most extensively characterized RND efflux pump) and proposed a mechanism for substrate binding and extrusion by this transporter. The RND transporter (AcrB) is assembled as an asymmetric trimer of which the individual AcrB monomers, known as protomers, individually undergo conformational changes during transport. As mentioned earlier, each individual AcrB protomer has 12 TMS (anchored in the cytoplasmic membrane) and 2 large periplasmic loops; the TMS do not interact with each other whereas the periplasmic loops interact both with one another as well as with the MFP (AcrA) and the OMP (TolC). The OMP, TolC, forms a barrel shaped trimer which creates a conduit spanning from the top of AcrB through the outer membrane. The MFP, AcrA, is oligomeric and while its exact function is unknown, it is thought to be a flexible

stabilizer of the AcrB/TolC interaction. **Figure 2-3** is a cross sectional depiction of the AcrAB-TolC complex.

The AcrB trimer is asymmetrical because each individual protomer is in a different conformation. The three conformations are known as binding, access, and extrusion. The protomer in the binding conformation has a large flexible binding pocket open to the periplasm, where substrate uptake is proposed to take place; this is depicted as the white spot in the middle of the purple AcrB protomer in **Figure 2-3**. It is unclear how the binding pocket of AcrB is able to accommodate the multitude of structurally distinct compounds known to be its substrates. The binding protomer is not open to the TolC exit conduit, however, it does undergo a conformational change during which the binding pocket collapses and is no longer open to the periplasm; this conformation is called the extrusion protomer. In the extrusion protomer, the light purple region of AcrB (adjacent to the aqua colored channel of TolC) relaxes and the substrate can move from AcrB through TolC to the extracellular space. The third protomer, access, can be imagined as an intermediate between binding and extrusion where the binding pocket is open to neither the periplasm nor TolC.

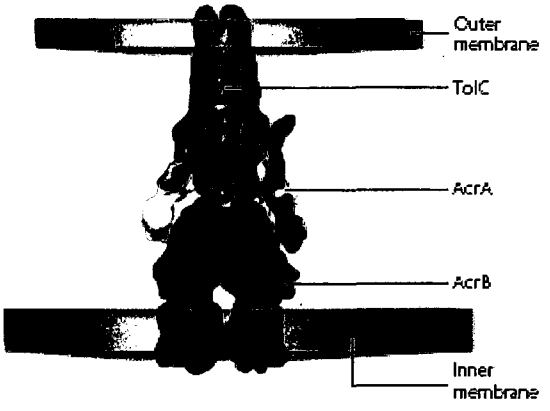

Figure 2-3 Cross sectional schematic of the AcrAB-TolC complex [taken from (51)]

These conformational changes are thought to be driven by a combination of substrate binding and protonation of key residues in the TMS of each AcrB protomer. The three protomers cycle through these three phases such that no two protomers are in the same conformation simultaneously (51, 66). Due to the high degree of homology between RND transporters, MFP, and OMP across species, it is likely that other RND pumps function in a manner similar to that proposed for AcrAB-TolC.

2.3 RND Efflux in *B. pseudomallei*

A recent Pathema (http://pathema.jcvi.org/Pathema) search of the *B. pseudomallei* K96243 genome revealed 34 proteins (both putative and characterized) involved in the transport of antibiotics. This included 9 ABC transporters, 1 MATE transporter, 13 MFS transporters, 1 SMR transporter, and at least 10 RND transporters. To date, only three *B. pseudomallei* efflux pumps have been characterized (AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC) all belonging to the RND family of transporters. The genetic structure of the three characterized and seven putative RND efflux pumps of *B. pseudomallei* K96243 are illustrated in **Figure 2-4**.

Seven of the ten RND pumps of *B. pseudomallei* are encoded by chromosome 1 and the remaining three are encoded by chromosome 2. Four of the pumps have the typical RND genetic organization, coding the MFP, RND transporter, and OMP in that order in a single operon. Similar to other heavy metal RND efflux pumps *czcC-czcB-czcA* and *BPSL0307-BPSL0308-BPSL0309* both have the OMP at the beginning of the operon instead of at the end. Those RND efflux pumps involved in extruding heavy metals do not usually also convey multidrug resistance. Uniquely, the *BPSL1268-BPSL1267-BPSL1266* operon encodes two RND transporters. Two pump operons,

BPSL1268-BPSL1267-BPSL1266 and *BPSL2234-BPSL2235,* do not include a gene encoding for the OMP as part of the operon, however, as mentioned earlier, these pumps may recruit an OMP transcribed from another operon.

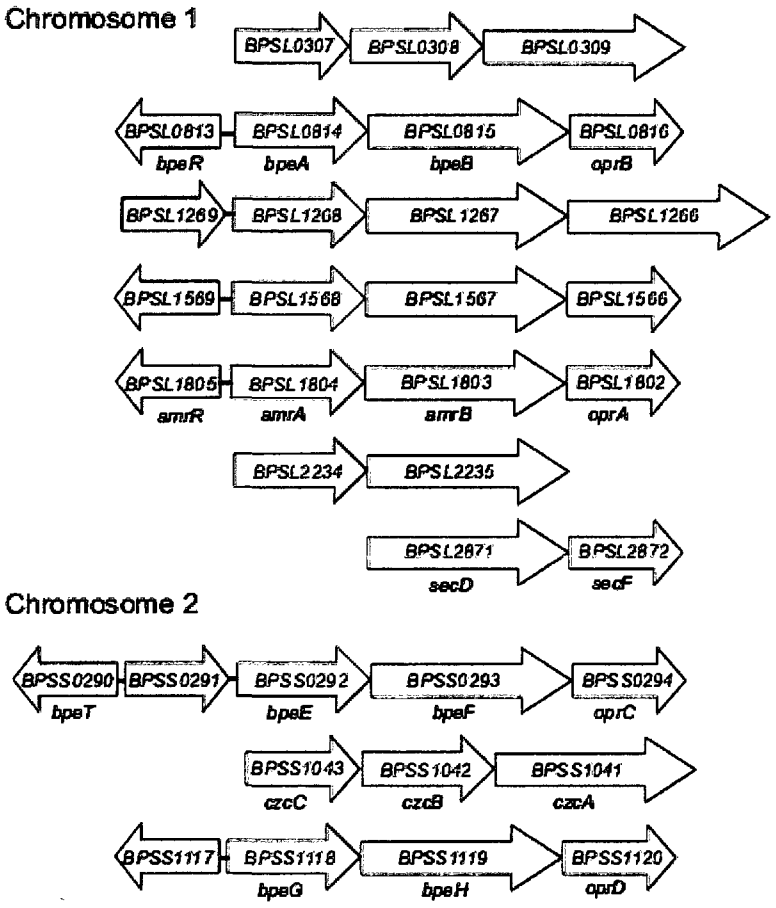

Figure 2-4 Genetic arrangement of putative and characterized RND efflux pumps from *B. pseudomallei* K96243 [taken from (41)]. RND transporter genes are represented by blue arrows, OMP genes by green, and MFP by yellow. When present, the local operon regulator genes are depicted as orange arrows. The BPSL number inside the arrow is the locust tag identifier. If known, the gene names have been included below their representative arrows.

2.3.1 AmrAB-OprA

The first *B. pseudomallei* efflux pump to be characterized, AmrAB-OprA was identified by Moore et al. as a major contributor to intrinsic streptomycin resistance using transposition mutagenesis of strain 1026b (62). This first report also stated that other aminoglycosides including kanamycin, tobramycin, and gentamicin as well as the

macrolides erythromycin and clarithromycin were substrates for AmrAB-OprA. However, clindamycin, ampicillin, ceftazidime, cetrimide, chloramphenicol, ciprofloxacin, nalidixic acid, rifampin, trimethoprim, and polymixin B were not apparent substrates for this pump in this report. As demonstrated by *lacZ* transcriptional fusion assays, *amrAB-oprA* is constitutively expressed at some level and a higher level of transcription is not induced by either streptomycin or stress conditions.

Work described in Chapter 5 demonstrated that expression of AmrAB-OprA in a surrogate *B. thailandensis* strain deficient in both AmrAB-OprA and BpeAB-OprB, revealed several more substrates for AmrAB-OprA including: norfloxacin, chloramphenicol, tetracycline, doxycycline, and acriflavine. Some of these substrates were not recognized by Moore et al. because they were not working in a BpeAB-OprB deficient background and substrate overlap between BpeAB-OprB and AmrAB-OprA masked the extrusion of some substrates. Furthermore, the same results were observed in a similar fashion in *B. pseudomallei* directly (T. Mima and H.P. Schweizer, unpublished results).

There is a putative TetR family regulatory protein, AmrR, encoded immediately upstream and divergently transcribed from the *amrAB-oprA* operon. Based on homology to other RND regulators, it is speculated that AmrR acts as a repressor of *amrAB-oprA* (62). However, there is currently no experimental data demonstrating the regulation of *amrAB-oprA* by AmrR or any other regulator.

2.3.2 BpeAB-OprB

BpeAB-OprB was identified by first constructing a genomic library with *B. pseudomallei* strain ATCC 23343 and subsequently probing the library with radiolabeled

probes specific to *ceoA* and *ceoB* (CeoAB-OpcM is a RND efflux pump from *Burkholderia cepacia*). The clones identified with these probes were sequenced and when aligned with the *B. pseudomallei* K96243 genome (which apparently became available during the course of this work) they mapped to a locus separate from that of the previously identified *amrAB-oprA*. Thus, Chan et al. had identified a new RND efflux pump in *B. pseudomallei* which they named BpeAB-OprB (15). Testing of a *bpeAB* deletion mutant in *B. pseudomallei* strain KHW revealed identical substrate profiles for AmrAB-OprA and BpeAB-OprB, with the exception of spectinomycin and clarithromycin which are substrates for AmrB but not BpeB (15).

Once again, work detailed in chapter 5 demonstrates a difference in substrate profile than what has been reported by Chan et al. When MICs of *B. thailandensis amrAB-oprA* mutants are compared to those of *B. thailandensis amrAB-oprA, bpeAB-oprB* double mutants it is clear that while macrolides, quinolones, lincosamides, and tetracyclines are all substrates for *bpeAB-oprB*, aminoglycosides are not substrates. Furthermore, the same results were observed in a similar fashion in *B. pseudomallei* directly (T. Mima and H.P. Schweizer, unpublished results).

A TetR family regulator, *bpeR*, is located directly upstream and transcribed divergently from *bpeAB-oprB*. *bpeAB-oprB* is constitutively expressed at some level, however, Chan and Chua demonstrated (by both RT-PCR and phenotypically by MIC) that deletion of *bpeR* resulted in overexpression of *bpeA* and, likewise, overexpression of *bpeR* resulted in complete repression of *bpeA* expression (13). Additionally, they demonstrated that *bpeAB-oprB* expression is growth phase dependent, induced by at least one of its substrates (erythromycin), has a secondary mechanism of regulation (perhaps

induction by the quorum sensing molecule, C8 homoserine lactone, C8HSL), and that BpeAB-OprB is involved in the extrusion of C8HSL (13). Because of its proposed role in quorum sensing, in addition to conveying multidrug resistance, BpeAB-OprB may play a significant role in the virulence of *B. pseudomallei.*

2.3.3 BpeEF-OprC

Cloned from a *B. pseudomallei* 1026b fosmid clone, BpeEF-OprC was characterized in surrogate *P. aeruginosa* and *B. thailandensis* strains (40). *bpeEF-oprC* was inserted in single copy into the chromosome of these surrogate strains. Expression of *bpeEF-oprC* was under control of the inducible *tac* promoter. Upon induction, it was clear that chloramphenicol and trimethoprim were substrates for this pump.

A LysR family transcriptional regulator is located directly upstream of and transcribed divergently from *bpeEF-oprC.* The function of this regulator as well as whether *bpeEF-oprC* is 'silent' or expressed constitutively in *B. pseudomallei* remains unknown, but is currently being elucidated in our lab. This information would be helpful in determining the potential for this pump to convey high level resistance, as both chloramphenicol and trimethoprim are used clinically for the treatment of melioidosis.

Uniquely, the operon encoding *bpeEF-oprC* also encodes a putative lipase, *BPSS0291* (40). The function of this lipase is unclear, however, once elucidated it may provide some insight on the physiological role of BpeEF-OprC, which, as with all other RND pumps, is unlikely to be solely a mechanism of antibiotic resistance.

2.3.4 Putative pumps

Of the seven putative pumps illustrated in **Figure 2-4**, *BPSL2234-BPSL2235, BPSL2871-BPSL2872,* and *BPSS1043- BPSS1042-BPSS1041* are unlikely to be involved

in multidrug efflux; more probable roles are cation efflux, general protein secretion, and heavy metal export, respectively (41). Regardless, the protein sequences of RND transporters from all 10 predicted RND operons in *B. pseudomallei* were aligned with protein sequences from RND transporters of other Gram negative bacteria to determine if sequentially related pumps shared the same substrate profiles. This alignment was accomplished using CLUSTALW and is represented as a phylogenetic tree in **Figure 2-5.** A list of the RND transporter proteins and their respective substrates is presented in **Table 2-4.**

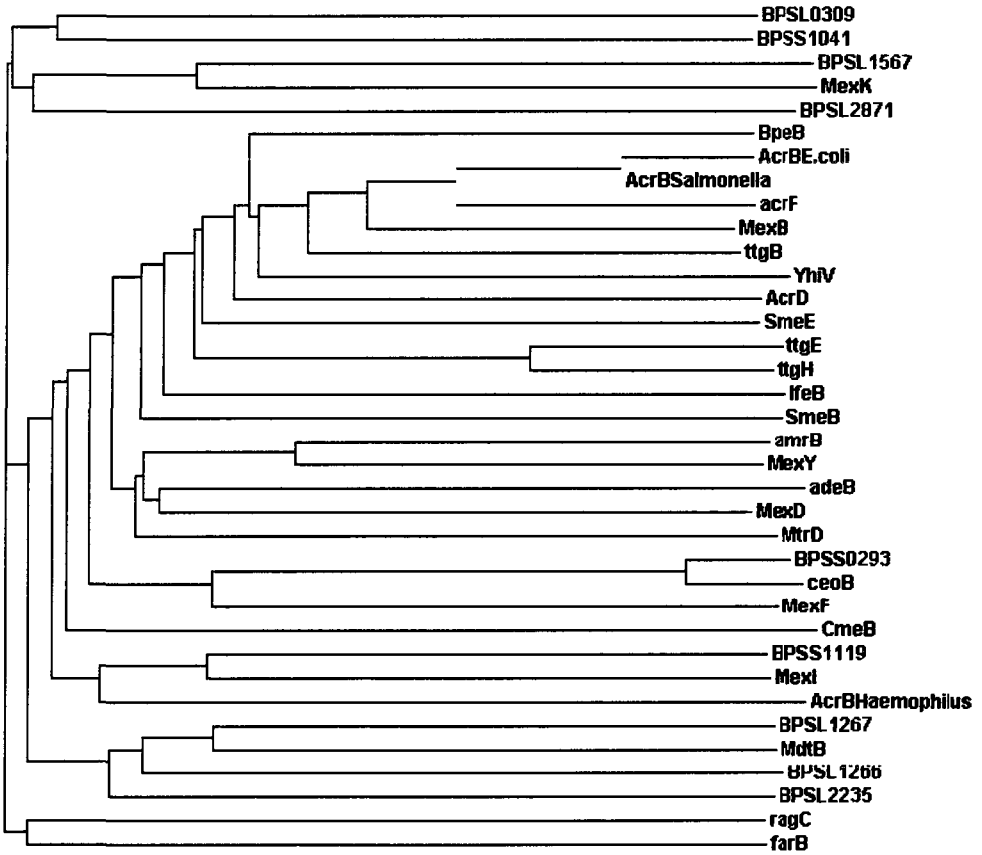

Figure 2-5 Phylogenetic tree representing relationship of *B. pseudomallei* RND transporter protein sequences to the protein sequence of characterized RND transporters from other Gram negative bacteria. *B. pseudomallei* RND transporters are highlighted in yellow. Refer to **Table 2-4** for abbreviations and protein orgins.

Organism	RND Transporter	Substrates(s)
Burkholderia pseudomallei	AmrB	NF, AG, ML, CM, TC, AC
	BpeB	ML, Q, LN, TC
	BpeF (BPSS0293)	CM, TM
	BPSL0309	NI
	BPSS1119	NI
	BPSL2235	NI
	BPSS1041	NI
	BPSL2871	NI
	BPSL1266	NI
	BPSL1267	NI
	BPSL1567	NI
Acinetobacter baumannii	AdeB	AG, CM, EB, FQ, NO, TC, TM
Agrobacterium tumefaciens	IfeB	COUMESTROL
	AmeB	CB, DC, NO, SDS
Bradyrhizobium japonicum	RagC	NI
Burkholderia cepacia	CeoB	CM, FQ, TM
Campylobacter jejuni	CmeB	AP, CM, CT, EB, EM, NA, FQ, PR, RF, TC
Escherichia coli	AcrB	AC, BL, BS, CM, CV, EB, FA, ML, NO ,OS, RF, TX
	AcrD	AG, DC, FU, NO
	AcrF	AC, BL, BS, CM, CV, EB, FA, ML, NO, OS, RF, TX
	MdtB	DC, NO
	MdtC	DC, NO
	YhiV	DC
Haemophilus influenzae	AcrB	AC, CV, EB, EM, NO, RF, SDS
Neisseria gonorrhoeae	MtrD	EB, FA, TX
	FarB	FA, TX
Pseudomonas aeruginosa	MexB	AC, AG, BL, CM, CV, EB, ML, NO, OS, SDS, SF, TC, TM, TR
	MexD	CM, CP, FQ, TC, TR
	MexF	CM, FQ
	MexY	AG, ML, TC
	MexI	Vanadium
	MexK	EM, TC, TR
P. putida	TtgB	OS
	TtgE	OS
	TtgH	OS
Stenotrophomonas maltophilia	SmeB	AG, BL, FQ
	SmeE	EM, FQ, OS, TC
Salmonella typhimurium	AcrB	BL, FQ

The phylogram and the pump substrate profiles in **Table 2-4** demonstrate that closely related pumps share similar substrate profiles. For example, in terms of protein sequence, BpeEF-OprC from *B. pseudomallei* is closely related to *B. cepacia* CeoAB-OpcM and chloramphenicol and trimethoprim are substrates for both pumps. Similarly, *B. pseudomallei* AmrAB-OprA and BpeAB-OprB are closely related to *P. aeruginosa* MexAB-OprM and MexXY-OprM, respectively and, with the exception of β-lactams, these pumps also share similar substrates. Thus, the substrate profile of putative *B. pseudomallei* could be predicted by comparison to related pumps. For instance, BPSS1119 may extrude vanadium, which is a substrate for the related *P. aeruginosa* pump, MexHI-OpmD.

2.4 Effects of RND efflux on diagnosis and treatment of melioidosis

Both diagnosis and treatment of melioidosis are complicated by the high intrinsic antibiotic resistance of *B. pseudomallei.* As discussed in chapter 1, the gold standard for diagnosis of melioidosis is culture of *B. pseudomallei* on Ashdown's agar which relies heavily on the intrinsic resistance of this organism to the aminoglycoside, gentamicin. Moore et al. have demonstrated that, at least in strain 1026b, gentamicin resistance is achieved through efflux by AmrAB-OprA (62). However, there are several reports of clinical *B. pseudomallei* isolates that are exquisitely susceptible to gentamicin; which, without other diagnostic methods, would not have been identified by isolation on Ashdown's agar (31, 80). Currently the rate of gentamicin susceptibility among *B. pseudomallei* clinical isolates in northeastern Thailand is approximately 1/1000, however, this is likely to be a very conservative estimate as many facilities are completely reliant

on culture of *B. pseudomallei* on Ashdown's agar for identification, and gentamicin susceptible isolates are excluded by this diagnostic method (80). Characterization of the gentamicin susceptibility of these clinical isolates as well estimation of the expression of *amrAB-oprA* in both clinical and environmental *B. pseudomallei* populations will help to address whether reliance on culture of this organism using Ashdown's agar is an appropriate diagnostic tool. These tasks and their results are described in chapters 3 and 4, respectively.

Treatment of melioidosis has been restricted to a few antibiotic options as a result of the intrinsic resistance of *B. pseudomallei* to most clinically relevant antibiotics (19, 87). In fact treatment of melioidosis is almost strictly limited to ceftazidime or carbapenems for the intensive phase and co-trimoxazole for the eradication phase. Acquired resistance to these antibiotics has been described, however, the mechanisms of such resistance remain unclear (20, 34, 95, 99). The fact that RND efflux can lead to high level resistance and pumps closely related to those found in *B. pseudomallei* convey resistance to ceftazidime, sulfonamides, and trimethoprim simultaneously, it is possible that a single determinate could lead to resistance to all possible treatment options. In order to fully understand this possibility, further characterization of RND efflux systems in *B. pseudomallei* is necessary.

2.4.1 Efflux pump inhibitors

Efflux pumps are attractive drug targets because the can potentiate the effects of established antibiotics in otherwise resistant strains thus overcoming not only acquired resistance but also intrinsic resistance (56). This realization has lead to a concerted effort to identify inhibitors of efflux pumps, which can be administered in combination with the

antibiotics to which these pumps confer resistance, much like a β-lactamase inhibitor is given in combination with some β-lactams (56). A schematic conceptualizing the use of an efflux pump inhibitor (EPI) to potentiate the efficacy of an antibiotic is provided in **Figure 2-6.** Furthermore, sub-inhibitory concentrations of antibiotic, facilitated by efflux, allow time for mutations leading to other mechanisms of resistance; therefore, the use of efflux pump inhibitors could potentially hinder the development of resistance by preventing sub-inhibitory intracellular antibiotic concentrations (50, 93). Most EPIs are identified through screening chemical libraries, screening compounds which are known eukaryotic efflux pump inhibitors, testing other drugs which are not antimicrobial but may inhibit efflux, or by modifying current antibiotics so they are still effective but are no longer substrates for efflux (56).

There are at least four possibilities for the mode of action of EPIs: inhibition by EPI binding directly to the pump (either irreversibly or by competitive inhibition), inhibition of the pump's energy source, and (depending on the class of pumps) interfering with the assembly of pump components, or blocking the outer membrane channel (42, 51). EPI mode of action is not well defined, as even the mechanisms of substrate efflux are still being elucidated, however, there is some evidence to support the first two possible modes of action. Although one group studying competitive inhibition of erythromycin from AcrAB-TolC in *E. coli* using steroid hormones (which are known to be excellent substrates for this pump) as the competitive substrate found that competitive inhibition to the point of erythromycin susceptibility was not possible (23). They conclude that perhaps the ability of AcrB to handle multiple substrates simultaneously reduces the possibility for competitive inhibition. There is no experimental data

supporting the last two possibilities for mode of action; they are simply derived by the knowledge that, in certain classes of tripartite pumps, all three parts must be present for function and that in these same types of pumps the substrate exits the cell through the outer membrane channel (51).

Screening of EPIs, to identify compounds which could potentiate the use of fluoroquinolones in *P. aeruginosa* (from efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN) as well as the AcrAB-TolC pump of *E. coli*, identified a synthetic compound (MC-207,110) which could potentiate the effects of some, but not all of the antibiotics extruded by these pumps (50). Researchers were able to show that MC-207, 110 did not interfere with the energy source for the pumps (the proton motive force) and thus, they hypothesized that the EPI was binding to the pump and selectively inhibiting efflux, although they had no evidence for this mode of action (50, 91). Recently, crystal structures of the AcrB transporter from the *E. coli* AcrAB-TolC efflux pump have been elucidated without substrate, as well as with two different antibiotic substrates (51). These structures, along with mutagenesis data implicating specific residues in binding of substrates with their pumps, lead researchers to purpose that some efflux pumps have several different substrate binding sites, lending support to the theory that EPIs could bind to an efflux pump and selectively, as well as competitively, inhibit efflux of particular substrates (49, 51).

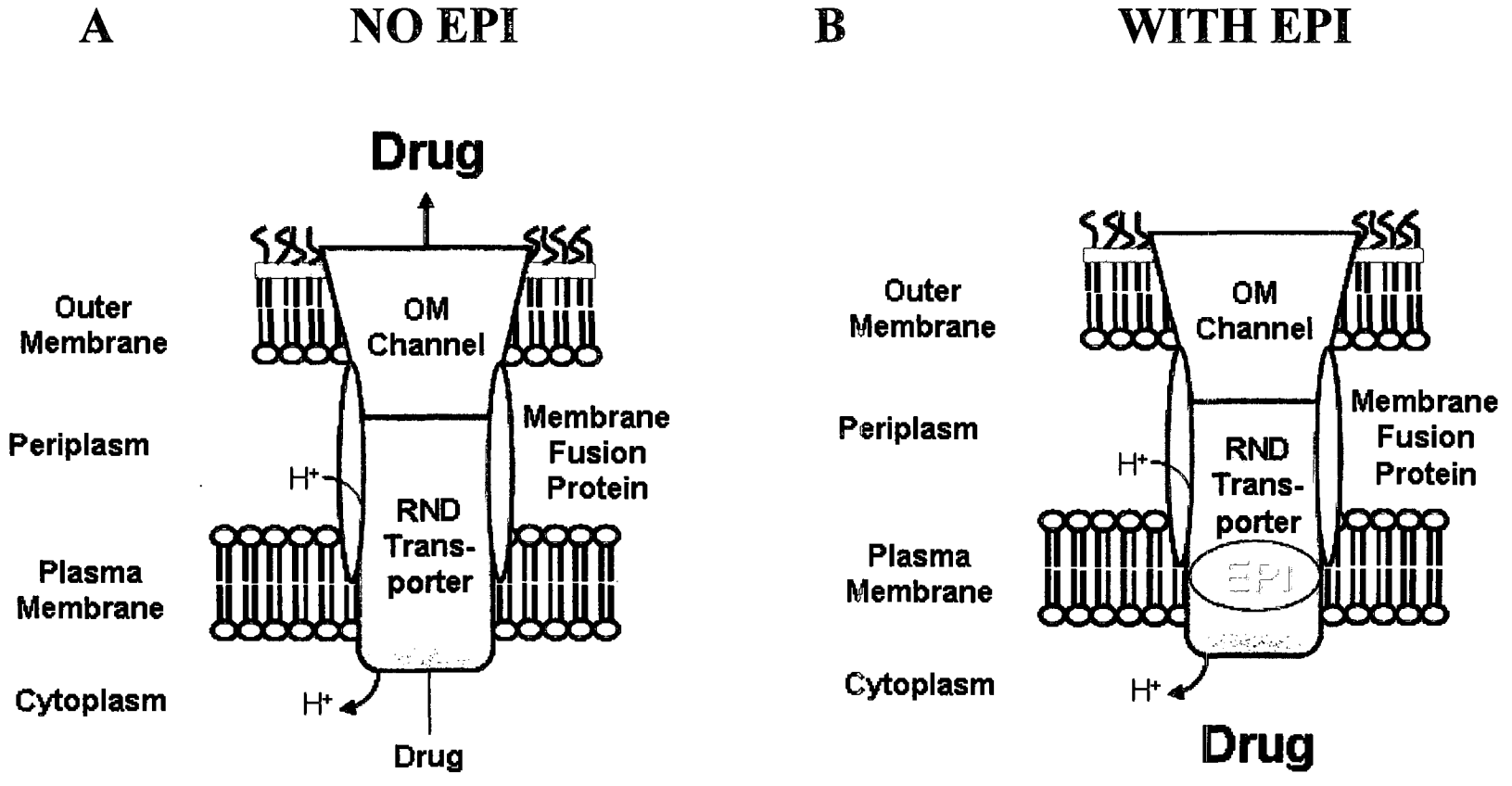

Figure 2-6 Efflux pump inhibitor effect on intracellular drug concentration [taken from (42)]. **(A)** In the absence of an efflux pump inhibitor, the drug (which is a substrate for the pump) is extruded from the cell and inhibitory concentrations are not achieved intracellularly, leading to drug resistance. **(B)** In the presence of the efflux pump inhibitor, drug extrusion is prevented, therefore the drug accumulates intracellularly and susceptibility can be achieved.

Another approach to developing EPIs has been to modify known substrates which presumably bind to the efflux pump, for which their parent molecules are substrates, and prevent binding of the normal substrate in either a competitive or non-competitive manner (55). This approach has been used in targeting the Tet drug transporters of *S. aureus* and *E. coli*. In this case several different modifications of tetracycline were created and tested until one was designed that blocked efflux, 13-cyclopentylthio-5-OH-tetracycline (13-CPTC) (42, 56, 91). This modified tetracycline has decreased antimicrobial activity (over tetracycline) but when given in combination, potentiates the activity of doxycycline (42).

Omeprazole, an anti-ulcer drug and eukaryotic efflux inhibitor, was identified in a screen of pre-existing drugs for EPI activity to potentiate fluoroquinolone activity in *S. aureus,* where fluoroquinolones are NorA efflux pump substrates (93). However, use of this drug as an EPI may not be feasible because the minimum potentiating concentration is much higher than the normal dose, so there is currently an effort to modify this drug to make it a physiologically feasible candidate for use as an EPI (93). This drug's mode of action in eukaryotic cells is inhibition of a proton pump but experimental data in *S. aureus* indicate that there is some other mode of action in prokaryotic cells (yet to be described) because it does not interfere with the proton gradient in this case (93).

Currently, EPI/antibiotic combinations are not used clinically, but the need for such a product is recognized and as a result there are ongoing collaborative efforts to design such a product (49, 53). *In vivo* data using a mouse model demonstrated the usefulness of MC-207, 110 in potentiating the activity of fluoroquinolones against resistant *P. aeruginosa* (49). However, attributes of this compound's chemical structure have

pharmacokinetic and toxicological implications leading to uncertainty as to the clinical use of this EPI (49). Development of EPIs is expensive, due to the nature of combination therapy, but development of a single EPI, given that it was broad-spectrum, could enable the use of many different antibiotics against numerous important bacterial pathogens which overshadows the cost and effort it would take to develop new antibiotics to address each resistant pathogen individually (51).

Efflux has proven to be a significant contributor to the intrinsic resistance of *B. pseudomallei*, specifically in regard to aminoglycosides, macrolides, lincosamides, tetracyclines, chloramphenicol and the quinolones (15, 40, 62). If resistance by efflux could be circumvented, these antibiotics would increase the number of possible treatment options and perhaps exceed the limitations of current treatment options due to their availability in oral formulations (which decreases cost and increases treatment feasibility) and increased cellular penetration (which may be more effective in killing intracellular *B. pseudomallei*). Chan and colleagues have investigated phenothiazines as a potential EPI against AmrAB-OprA and BpeAB-OprB in *B. pseudomallei* (14). Their findings indicate potentiation of some aminoglycosides and macrolides when given in combination with phenothiazines (14). The mechanism by which phenothiazines inhibit efflux may include disruption the proton gradient which powers RND efflux (37). Disruption of the proton gradient is not an optimal mechanism for efflux pump inhibition, as eukaryotic cells also utilize a proton gradient and interruption of such would not confer selective toxicity. Therefore, the search for EPIs with clinical applications for potentiating antibiotic efficacy in *B. pseudomallei* is still relevant. A tool for screening compounds which function as EPIs in *B. pseudomallei* is described in chapter 5.

2.5 Hypothesis, aims, and preview of chapters

Efflux by the RND family of efflux pumps has been described as a significant contributor to both intrinsic and acquired resistance of many Gram negative pathogens including *Pseudomonas aeruginosa, Escherichia coli,* and *Acinetobacter baumanii,* just to name a few. The *B. pseudomallei* genome encodes at least 10 RND efflux systems; of the three pumps that have been characterized, all have demonstrated capacity as high level multidrug resistance determinants. Further characterization of these systems would facilitate the improvement of current treatment strategies which are expensive and would be unrealistic options in the event of a large scale intentional release of this pathogen. There are several questions that need to be addressed as characterization of these efflux systems continues including:

- Why do some strains display exquisite susceptibility to antimicrobials that are known substrates for constitutively expressed RND efflux pumps?
- RND efflux pumps of *B. pseudomallei* have only been characterized in two strains of the organism. In an organism infamous for its unusual level of genomic plasticity, is the level of efflux pump expression consistent across a representative population of strains?
- Does efflux pump expression correlate to resistance to clinically relevant antimicrobials in a representative collection of *B. pseudomallei* strains?
- Manipulation of this organism must be conducted in a BSL3 laboratory by individuals with FBI approval. Facilities with small molecule libraries (SML) (which can be screened for potential efflux pump inhibitors) cannot necessarily meet the standards for working with *B. pseudomallei.* Can a surrogate organism

be used for screening SMLs for compounds that inhibit *B. pseudomallei* RND efflux pump under BSL2 conditions?

Thus, the hypothesis for this work is that, in *B. pseudomallei,* RND pump-mediated efflux is a significant antibiotic resistanance determinant and strains lacking efflux pump expression become susceptible to clinically relevant antimicrobials. Therefore a system to identify inhibitors of efflux would facilitate treatment of *B. pseudomallei* infections. In order to test this hypothesis, the following specific aims were pursued:

I.) Characterization of the mechanism for gentamicin susceptibility observed in some *B. pseudomallei* strains (chapter 3).

II.) Use of quantitative real-time PCR (*q*RT-PCR) to compare RND efflux pump expression in a large collection of both clinical and environmental *B. pseudomallei* strains (chapter 4).

III.) Correlate efflux pump expression and antimicrobial resistance by comparing expression (as measured by *q*RT-PCR) and resistance (as measured by minimal inhibitory concentration (MIC)) in a large collection of both clinical and environmental *B. pseudomallei* strains (chapter 4).

IV.) Create a *B. thailandensis* surrogate strain background which expresses *B. pseudomallei* RND efflux pumps and can be used for discovery of efflux pump inhibitors under BSL2 conditions (chapter 5).

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CHAPTER 3

MOLECULAR BASIS OF AMINOGLYCOSIDE AND MACROLIDE SUSCEPTIBILITY OF RARE *Burkholderia pseudomallei* CLINICAL ISOLATES FROM THAILAND: IMPLICATIONS FOR PATHOGENESIS

The work presented in this chapter demonstrates that naturally occurring aminoglycoside susceptibility in *B. pseudomallei* occurs as a result of insufficient expression or deletion of *amrAB-oprA*. I acknowledge the contribution of Katie Propst, who conducted all of the animal experiments.

3.1 Abstract

Burkholderia pseudomallei is intrinsically resistant to aminoglycosides and macrolides, mostly due to AmrAB-OprA efflux pump expression. We investigated the molecular mechanisms of aminoglycyoside susceptibility exhibited by Thai strains 2188a, 3799a, and 708a. qRT-PCR revealed absence of *amrB* transcripts in 708a and greatly reduced levels in 2188a and 3799a. Serial passage on increasing gentamicin concentrations yielded 2188a and 3799a mutants that became simultaneously resistant to other aminoglycosides and macrolides, whereas such mutants could not be obtained with 708a. Transcript analysis showed that the resistance of the 2188a and 3799a mutants was due to upregulation of *amrAB-oprA* expression by unknown mechanism(s). Use of a

PCR walking strategy revealed that the *amrAB-oprA* operon was missing in 708a and that this loss was associated with deletion of more than 70 kb of genetic material. Rescue of the *amrAB-oprB* region from a 708a fosmid library and sequencing showed the presence of a large chromosome 1 deletion (131 kb compared to K96243 and 141 kb compared to 1710b). This deletion not only removed the *amrAB-oprA* operon, but also the entire gene clusters for malleobactin and cobalamin synthesis. Other genes deleted included the anaerobic arginine deiminase pathway, putative type 1 fimbriae and secreted chitinase. Despite missing several putative virulence genes, 708a was fully virulent in a murine melioidosis model, yet very susceptible to aminoglycosides. Strain 708a may be a natural candidate for genetic manipulation experiments that use Select Agent compliant antibiotics for selection and validates the use of laboratory-constructed Δ(*amrAB-oprA*) mutants in such experiments. Because 708a was isolated from a splenic abscess and was therefore capable of growing under presumably anaerobic conditions, studies of these rare Thai isolates have provided insights into the pathogenesis and pathophysiology of *B. pseudomallei* infections.

3.2 Introduction

Melioidosis is a disease caused by *Burkholderia pseudomallei* (8, 41). Melioidosis is endemic to tropical and subtropical regions of the world (14) and is considered an emerging disease (e.g. NE Thailand (32)) as well as a disease of biodefense importance (32). Melioidosis has received worldwide popular attention in the wake of the 2004 Southeast Asia Tsunami disaster (2, 9, 12, 33). Treatment of melioidosis is complicated by the intrinsic resistance of *B. pseudomallei* to many antibiotics, including aminoglycosides, macrolides, several penicillins, and first and second generation

cephalosporins (8, 41, 42). Factors complicating drug therapy are the ability of *B. pseudomallei* to form biofilms (38) and to enter into prolonged, presumably intracellular, latency periods of up to 6 decades (25).

Genome sequence analysis has provided an indication of possible mechanisms of resistance to antimicrobial compounds, but less than a handful of resistance genes have been experimentally confirmed to date (16). The K96243 and other *B. pseudomallei* genomes encode an arsenal of efflux pumps, including 10 pumps belonging to the resistance nodulation cell division (RND) family, which play major roles in clinically significant antibiotic resistance in Gram-negative bacteria. Two of these, AmrAB-OprA (24) and BpeAB-OprB (7) were reported to play major roles in high-level resistance to aminoglycosides and macrolides, but our unpublished results with strain 1026b indicate that BpeAB-OprB does not efflux aminoglycosides. Using a surrogate *Pseudomonas aeruginosa* strain we recently showed that BpeEF-OprC extrudes chloramphenicol and trimethoprim (17). While the majority of clinical *B. pseudomallei* isolates exhibit high levels of aminoglycoside and macrolide resistance, rare (~1:1000) isolates are susceptible to these antibiotics. It has been noted that the resistance profile of these isolates matches that of *amrAB-oprA* mutants suggesting possible involvement of AmrAB-OprA in intrinsic aminoglycoside and macrolide resistance or lack thereof (30), but this has not yet been experimentally demonstrated. In this report we provide evidence that the susceptibility of three isolates from Northeastern Thailand is indeed due to lack of, or greatly reduced, AmrAB-OprA expression, either due to deletion or unknown mechanisms. Furthermore, deletion of a >130 kb region of chromosome 1 in one strain

not only removed *amrAB-OprA*, but also genes encoding several putative virulence factors and other functions implicated in bacterial pathogenesis and physiology.

3.3 Materials and methods

3.3.1 Bacterial strains, media and growth conditions.

B. pseudomallei strains used in this study are listed in **Table 3-1**. *Escherichia coli* strains used for cloning experiments were DH5α (20) or DH5α(λ*pir*) (laboratory strain). All bacteria were routinely grown with aeration at 37°C. Low salt (5 g/L NaCl) Lennox LB broth (LSLB) and agar (MO BIO Laboratories, Carlsbad, CA) were used as rich media. M9 medium (22) with 10 mM glucose was used as the minimal medium. Unless otherwise noted, antibiotics were added at the following concentrations: 100 μg/ml ampicillin (Ap), 12.5 μg/ml chloramphenicol (Cm), 15 μg/ml gentamicin (Gm), 35 μg/ml kanamycin (Km) and 25 μg/ml zeocin (Zeo) for *E. coli*; 1,000 μg/ml Km and 2,000 μg/ml Zeo for wild-type *B. pseudomallei* and 50 μg/ml for Gm susceptible *B. pseudomallei* strains. Antibiotics were either purchased from Sigma, St. Louis, MO (ampicillin, chloramphenicol, erythromycin, kanamycin, polymyxin B and streptomycin), EMD Biosciences, San Diego, CA (gentamicin), Invitrogen, Carlsbad, CA (zeocin) or Biomol via VWR International, West Chester, PA (spectinomycin).

3.3.2 DNA and genetic methods.

Published procedures were employed for manipulation of DNA, and transformation of *E. coli* and *B. pseudomallei* (10, 27, 28). Plasmid DNAs were isolated from *E. coli* and *B. pseudomallei* using the QIAprep Mini-spin kit (Qiagen, Valencia, CA). Colony PCR with *B. pseudomallei* was performed as previously described (10). Custom oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Isolation of chromosomally-integrated mini-Tn*7* elements followed by Flp-mediated selection marker excision was performed using recently published procedures (10). Quantitative real-time PCR was performed using the methods and primer sets described by Kumar et al. (18). Other primer sequences are shown in **Table 3-1**. Total RNA was extracted from cells grown to late log phase in LSLB medium using the RNeasy Mini Kit (Qiagen).

Table 3-1 Strains, plasmids and primers used in this study. [a]Abbreviations: AG, aminoglycosides; Ap, ampicillin; Gm, gentamicin; Km, kanamycin; ML, macrolides; r, resistance; Zeo, zeocin. [b]P_{tac}, *E. coli lac/trp* operon hybrid promoter. [c]Only selected primers are shown; other primer sequences are available from the authors upon request. Oligonucleotides were purchased from IDT, Coralville, IA.

Strain or plasmid	Relevant properties[a]	Reference or source
B. pseudomallei		
1026b	AG and ML resistant wild-type strain; clinical isolate	(15)
DD503	AG and ML susceptible Δ (*amrR-amrAB-oprA*)1026b derivative	(24)
708a	AG and ML susceptible clinical isolate	(30)
2188a	AG and ML susceptible clinical isolate	(30)
3799a	AG and ML susceptible clinical isolate	(30)
Bp24	Spontaneous AG and ML resistant derivative of 3799a	This study
Bp35	Spontaneous AG and ML resistant derivative of 2188a	This study
Bp50	1026b with Δ (*amrR-amrAB-oprA*)	
Bp66	Low level Gm^r derivative of 708a	This study
Bp187	Bp24 with Δ (*amrR-amrAB-oprA*)	This study
Bp202	Bp187::mini-Tn*7*T-LAC	This study
Bp194	Bp187::mini-Tn*7*T-LAC-$amrA^+B^+$-$oprA^+$	This study

Bp192	Bp35 with Δ (*amrR-amrAB-oprA*)	This study
Bp201	Bp192::mini-Tn*7*T-LAC	This study
Bp200	Bp192::mini-Tn*7*T-LAC-*amrA*$^+$*B*$^+$-*oprA*$^+$	This study
Bp209	Bp24 with Δ*amrR*	This study
Plasmids		
pEX18Km-*pheS*	Km^r; gene replacement vector	(4)
pEX-S12*pheS*	Gm^r; gene replacement vector	Lopez and Schweizer, unpublished
pUC18T-mini-Tn*7*T-LAC	Ap^r, Gm^r; mini-Tn*7* cloning and delivery vector	(11)
pPS2142	Ap^r, Gm^r; pUC18T-miniTn*7*T-LAC with *amrA*$^+$*B*$^+$-*oprA*$^+$; *amrAB-oprA* expression under $P_{tac}{}^b$ control	(10)
pTNS3	Ap^r; source of Tn*7* transposase components TnsABCD	(10)
pFKM2	Ap^r Km^r; source *of FRT-nptII-FRT* cassette	(10)
pFLPe2	Zeo^r; source of Flpe recombinase	(10)
pPS1927	Ap^r; pWSK29 (39) with ~15 kb strain 1026b chromosomal *Eco*RI fragment containing *amrA*$^+$*B*$^+$-*oprA*$^+$	This study
pPS2282	Ap^r; pGEM-T Easy (Novagen) with ~3.1 kb PCR fragment containing Δ(*amrR-amrAB-oprA*)::*FRT-nptII-FRT*	This Study
pPS2354	Gm^r Km^r; pEX-S12*pheS* with ~3.1 kb blunt-ended *Eco*RI fragment of pPS2282 cloned into the *Sma*I site	This Study
pPS2408	Ap^r, Km^r; pCR2.1 (Invitrogen) with 806 bp *amrR* containing fragment	This study
pPS2415	Ap^r, Km^r; pPS2408 with 162 bp *Nru*I deletion within *amrR*	This study
pPS2439	Km^r; pEX18Km-*pheS* with 660 bp *Eco*RI fragment from pPS2415	This study
Primers^c		
597	5'-CGAATTGGGGATCTTGAAGTTCCT	This study
1546	5'-TACATGGCGATAGCTAGACTGG	This study

1599	5'-CGCGCGCAATTGTTCCTC	This study
1600	5'-TCGTAAGAAAGCGACACGCA	This study
1601	5'-CGATTCTTCGCGCGTCTTG	This study
1602	5'-CGCGTGCGTGCCCATTCG	This study
1742	5'-AAGACCGCGCTCTATTACGA	This study
1743	5'-TCGTCACCGTATCAGTGCAT	This study
1756	5'-ATCTTGCCGTTGAAGTGTCC	This study
1757	5'-ATCGCTGAACACGAAGAACC	This study
1774	5'-ACTAGTAGTGAGCGCAACGCAATTA	This study
1779	5'-GCCTCTTCGCTATTACGC	This study
1797	5'-GTTCGTCGCCGAGGAGT	This study
1801	5'-GAAGCCGGTGAAATCGACG	This study

3.3.3 Mutant construction.

For construction of a Δ*amrR* mutant, the *amrR* containing region was PCR amplified from *B. pseudomallei* strain 2188a genomic DNA using primers 1599 and 1600 and Platinum *Taq* HiFi DNA polymerase (Invitrogen, Carlsbad, CA). The resulting 806 bp fragment was purified from an agarose gel using the Fermentas DNA Extraction Kit (Glen Burnie, MD) and ligated to pCR2.1 (Invitrogen) to yield pPS2408. Next, a 162 bp *Nru*I fragment was deleted from within *amrR*, followed by religation of the resulting larger fragment including the pCR2.1 backbone. This step yielded pPS2415. Finally, pPS2439 was obtained by cloning a 660 bp *Eco*RI fragment from pPS2415 containing the Δ*amrR* allele and flanking regions into the *Eco*RI site of pEX18Km-*pheS* (4). For isolation of Δ(*amrR-amrAB-oprA*) mutants, three partially overlapping DNA fragments representing flanking DNA segments and the Km^r marker were PCR-amplified from 50 ng pPS1927 and pFKM2 (10) DNA templates and then spliced together by an overlap

extension PCR. To do this, the following fragments were amplified in a first-round PCR using the following primers: a 892-bp *amrR* upstream fragment using primers 1581 (5'-agggtgtccacatccttgaa) and 1582 (5'-TCAGAGCGCTTTTGAAGCTAATTCGgacacttcaacggcaagat), a 828-bp *oprA* downstream fragment using primers 1583 (5'-AGGAACTTCAAGATCCCCAATTCGgtcgccgaatacgagaagac) and 1584 (5'-gaaatacgccttgacgcact), and a 1382-bp *FRT-nptII-FRT* fragment using primers 596 (5'-CGAATTAGCTTCAAAAGCGCTCTGA) and 597 (5'-CGAATTGGGGATCTTGAAGTTCCT)(Lowercase letters denote chromosome-specific sequences and uppercase letters *FRT* cassette-specific sequences.) These fragments were combined in a second PCR and, after gel purification, the resulting recombinant ~3.1-kb DNA fragment was cloned into pGEM-T Easy (Novagen), which yielded pPS2282. The Δ(*amrR-amrAB-oprA*::*FRT-nptII-FRT*) cassette was excised from pPS2282 with *Eco*RI, blunted ended with T4 DNA polymerase (NEB) and ligated into the *Sma*I site of pEX-S12*pheS* (C. Lopez and H. Schweizer, unpublished) yielding pPS2354. Gene replacement using PheS-mediated counter-selection on M9-glucose supplemented with 0.15% *p*-chlorophenylalanine was performed as previously described (4) except that *E. coli* strains SM10(λ*pir*) or RHO1 (a Km susceptible derivative of SM10[λ*pir*](23); D. Rholl and H. Schweizer, unpublished) were used for conjugation experiments. The recipient strain was either Bp24 or Bp35 and merodiploids were selected on LSLB medium supplemented with 1000 μg/ml Km (to select for the pEX18Km-*pheS* backbone in case of Δ*amrR* or the Δ[*amrR-amrAB-oprA*::*FRT-nptII-FRT*] cassette cloned in pEX-S12*pheS*) and 100 μg/ml polymyxin B (to counterselect against RHO1). *p*-

chlorophenylalanine resistant colonies were then obtained and screened for the presence of the correct deletion alleles by colony PCR (10) and primers 1599 and 1600 for Δ*amrR* or primers 597 and 1546 for Δ (*amrR-amrAB-oprA*)::*FRT-nptII-FRT*. An unmarked Δ(*amrR-amrAB-oprA*) mutation was obtained after Flp recombinase- mediated excision of the *nptII* marker using pFlpe2 (10). The presence of the deletion allele was verified by phenotypic (Gm susceptibility) and genotypic (PCR with primers 1581 and 1584) analyses.

3.3.4 Fosmid library construction and screening.

Genomic DNA was extracted from strain 708a using the QiAmpDNA Mini Kit (Qiagen, Valencia, CA). Fosmids containing ~40 kb inserts were isolated using the CopyControl Fosmid Library Production Kit following manufacturer's instructions (Epicentre, Madison, WI). Approximately 1,200 Cm^r resistant colonies were pooled in groups of 30 (designated pools A-Z and 1-11), grown overnight in Cm containing medium, induced to high copy number and fosmid DNA was extracted using the QIAprep Mini-spin kit (Qiagen). Fosmid DNA from the 30 pools were screened by PCR using primers 1742 and 1743, and PCR products were obtained from 5 pools. DNA from these pools was transformed into *E. coli* DH5α and single colonies were screened for the presence of the correct clones by PCR using primers 1742 and 1743. DNA was extracted from these clones and sequenced with primers 1774 and 1779 which anneal in the fosmid backbone, as well as 1742 which anneals in the insert. Sequences obtained with primers 1774 and 1779 were BLAST searched against genome sequences of *B. pseudomallei* strains K96243, 1710b, 1106a and 668.

3.3.5 Isolation of gentamicin resistant mutants.

Gentamicin resistant derivatives of strains 2188a and 3799a were isolated in several steps. First, the strains were grown overnight at 37°C in LSLB medium containing 8 μg/ml Gm. The bacteria were then diluted into fresh LSLB medium containing 16 μg/ml Gm, followed by outgrowth at 37°C. The selection steps were repeated using LSLB medium containing 32, 64 and 128 μg/ml Gm. Similar selection steps were performed with 708a except that lower Gm concentrations of 2, 4, 8 and 16 μg/ml were employed.

3.3.6 Antimicrobial susceptibility testing.

Minimal inhibitory concentrations (MICs) were determined in Mueller-Hinton broth from Becton Dickinson (Franklin Lakes, NJ) by the two-fold broth microdilution technique following Clinical and Laboratory Standards Institute guidelines (13). The MICs were recorded after incubation at 37°C for 15 to 16 h.

3.3.7 Animal infection experiments.

Ethics Statement: All animal procedures were performed using standard protocols and according to guidelines approved by Colorado State University BioSafety Committee and the Institutional Animal Care and Use Committee. For animal infection experiments, *B. pseudomallei* strains were grown in LB medium to saturation by overnight incubation at 37°C with aeration. Glycerol was added to a final concentration of 15% and cell suspensions were stored at -80°C until ready for use. Inocula for *in vivo* infections were prepared by thawing and diluting the frozen bacterial stocks in sterile phosphate buffered saline (Sigma-Aldrich). Female BALB/c mice between 6-8 weeks of age were used for infection studies (Jackson Laboratories, Bar Harbor, ME). Mice were housed under pathogen-free conditions, and provided sterile water and food *ad libitum*. All animal infections were done using intranasal (i.n.) inoculation. Mice were anesthetized by

intraperitoneal injection of 100 μg/g body weight of ketamine (Fort Dodge Animal Health, Overland Park, KS) and 10 μg/g body weight of xylazine (Ben Venue Laboratories, Bedord, OH). For all infections, the desired inoculum of *B. pseudomallei* was suspended in phosphate buffered saline. The 20 μl inoculum volume was delivered i.n, with the dose split evenly between both nostrils. At the completion of challenge studies, animals were humanely euthanized, according to study endpoints approved by the Animal Care and Use Committee at Colorado State University.

3.4 Results and Discussion

3.4.1 Aminoglycoside and macrolide susceptible isolates show reduced or absent AmrAB-OprA expression.

In agreement with previously published results, the aminoglycoside and macrolide susceptibility patterns of strains 708a, 2188a and 3799a isolated from human patients with various disease manifestations and clinical outcome (**Table 3-2**) were similar to those observed with the AmrAB-OprA deficient strain DD503 (**Table 3-3**). Quantitative real-time PCR was therefore used to assess *amrAB-oprA* expression relative to strain 1026b known to express this efflux pump. No *amrB* transcripts were detected in strains 708a and strain DD503 (Δ*amrAB-oprA* control); *amrB* transcript levels were significantly lower in 2188a and 3799a than those measured in 1026b (**Figure 3-1**). As, in our hands, 2 to 3 fold differences in mRNA levels determined by *q*RT-PCR make the difference between low- and high-level RND pump-mediated resistance, these data support the notion that the aminoglycoside and macrolide susceptibilities of strains 708a, 2188a and 3799a are due to reduced or lack of AmrAB-OprA efflux pump expression.

Table 3-2 *B. pseudomallei* strains: origins, properties and clinical details. [a]MIC determinations were performed in Thailand using the E-test.

Strain	Isolation Date	Clinical Details	Gentamicin MIC[a]
708a	30.8.90	32 year old male; 21 days fever and 14 days abdominal pain. No risk factors for melioidosis. Splenic abscess as single infectious site. Splenectomy required to control infection. Treated with intravenous ceftazidime followed by oral doxycycline. Survived.	0.5 μg/ml
2188a	18.12.98	22 year old male rice farmer; 14 days fever, cough, sputum, swollen left knee. Known diabetic. Bacteremic with lung and joint involvement. Treated with joint washout and intravenous amoxicillin/clavulanic acid. Developed respiratory failure and died the day after admission.	0.5 μg/ml
3799a	12.12.05	66 year old female rice farmer; 15 days cough, breathlessness, sputum. History of chronic renal failure. Bacteremic with lung and renal involvement. Treated with ceftazidime. Died from septic shock 4 days after admission.	1 μg/ml

3.4.2 Gentamicin resistant derivatives of 2188a and 3799a, but not 708a, express AmrAB-OprA.

As we were able to PCR amplify the 5' and 3' regions of the *amrAB-oprA* operon from strains 2188a and 3799a, but not 708a (data not shown), we suspected that this operon was absent from 708a and present but expressed at low levels 2188a and 3799a. To test this hypothesis, we attempted to isolate Gm resistant derivatives of these strains. Highly (MIC >1024 μg/ml) Gm^r derivatives, e.g. Bp35 and Bp24, were readily obtained with strains 2188a and 3799a, but not with 708a (e.g. Bp66) (**Table 3-3**). Moreover, the Gm^r 2188a and 3799a derivatives Bp35 and Bp24 became simultaneously resistant to other aminoglycosides and macrolides and their antibiotic susceptibility profiles resembled that of AmrAB-OprA expressing strain 1026b (**Table 3-3**). In contrast, the moderately (MIC 32 μg/ml) Gm^r derivative of 708a (Bp66) did not simultaneously

Table 3-3 Antibiotic susceptibilities of *B. pseudomallei* strains. [a]Cla, clarithromycin; Cli, clindamycin; Ery, erythromycin; Gm, gentamicin; Spc, spectinomycin; Str, streptomycin. [b]ND, not done; DD503 is streptomycin resistant because of a chromosomal *rpsL* mutation. [c]The mini-Tn7 elements are integrated at the *glmS2*-associated Tn7 attachment site (10). MIC values were determined in cells grown in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside.

		MIC (μg/ml) for:					
Strain	**Known Genotype**	**Gm[a]**	**Str**	**Spc**	**Ery**	**Cla**	**Cli**
1016b	Wild-type	256	1024	512	128	64	>1024
DD503	1026b with Δ(*amrR-amrAB-oprA*)	2	ND[b]	64	8	4	>1024
708a		1	8	32	16	16	>1024
2188a		1	8	32	16	32	>1024
3799a		2	8	64	16	16	>1024
Bp24	Gm^r derivative of 3799a	>1024	1024	256	64	16	>1024
Bp35	Gm^r derivative of 2188a	>1024	>1024	>1024	256	512	>1024
Bp66	Low level Gm^r derivative of 708a	32	8	16	4	16	>1024
Bp187	Bp24 with Δ(*amrR-amrAB-oprA*)	2	16	128	16	16	>1024
Bp202	Bp187::mini-Tn*7*T-LAC[c]	4	32	128	8	16	>1024
Bp194	Bp187::mini-Tn*7*T-LAC-*amrA$^+$B$^+$-oprA$^+$*	>1024	>1024	>1024	256	512	>1024
Bp192	Bp35 with Δ(*amrR-amrAB-oprA*)	2	16	128	16	16	>1024
Bp202	Bp192::mini-Tn*7*T-LAC[c]	4	32	128	8	16	>1024
Bp200	Bp192::mini-Tn*7*T-LAC-*amrA$^+$B$^+$-oprA$^+$*	>1024	>1024	>1024	256	256	>1024
Bp209	Bp24 with Δ*amrR*	>1024	>1024	1024	128	32	>1024

become resistant to other aminoglycosides and erythromycin. None of the strains tested exhibited altered clindamycin resistance. Clindamycin is a substrate of BpeAB-OprB but not AmrAB-OprA (T. Mima and H. Schweizer, unpublished data). Consistent with these

observations, significantly increased *amrB* transcript levels were detected in Bp24 and Bp35 (**Figure 3-1**, panels **A** and **B**), but not Bp66 (not shown). Deletion of *amrAB-oprA* from Bp24 and Bp35 resulted in loss of aminoglycoside and macrolide resistance which could be complemented back to wild-type levels by a chromosomally integrated mini-Tn*7* expressing *amrA*$^+$*B*$^+$-*oprA*$^+$ (**Table 3-3**). Summarily, these results indicate that the *amrAB-oprA* operon is absent from 708a and present, but not expressed in sufficient levels in strains 2188a and 3799a to confer aminoglycoside and macrolide resistance.

3.4.3 Lack of AmrAB-OprA expression in 2188a and 3799a is not due to mutations in the *amrAB-oprA* regulatory region.

To assess whether lack of *amrAB-oprA* expression in strains 2188a and 3799a is due to mutations in the operon's regulatory region, the *amrR-amrA* intergenic region was amplified with primers 1601 and 1602 and sequenced. These analyses revealed that the sequence of the *amrR-amrA* intergenic regions of strains 2188a and 3799a and their Gm^r derivatives Bp35 and Bp24 were identical (data not shown). Since increased *amrAB-oprA* expression in strains Bp24 and Bp35 was accompanied by increased *amrR* expression (**Figure 3-1, panel C**), we tested whether AmrR may function as an activator of *amrAB-oprA* transcription. However, deletion of *amrR* from Bp24 had no effect on AmrAB-OprA expression as the MICs of Bp24 and its Δ*amrR* derivative Bp209 were indistinguishable (**Table 3-3**). Furthermore, amplification of the *amrR* coding sequences from 2188a and 3799a and their Gm^r derivatives Bp35 and Bp24 with primers 1599 and 1600 did not reveal any mutations in *amrR*. In summary, these data revealed that i) lack of AmrAB-OprA expression in 2188a and 3799a was not caused by mutations in the *amrAB-oprA* regulatory region and ii) increased *amrAB-oprA* expression in Gm^r

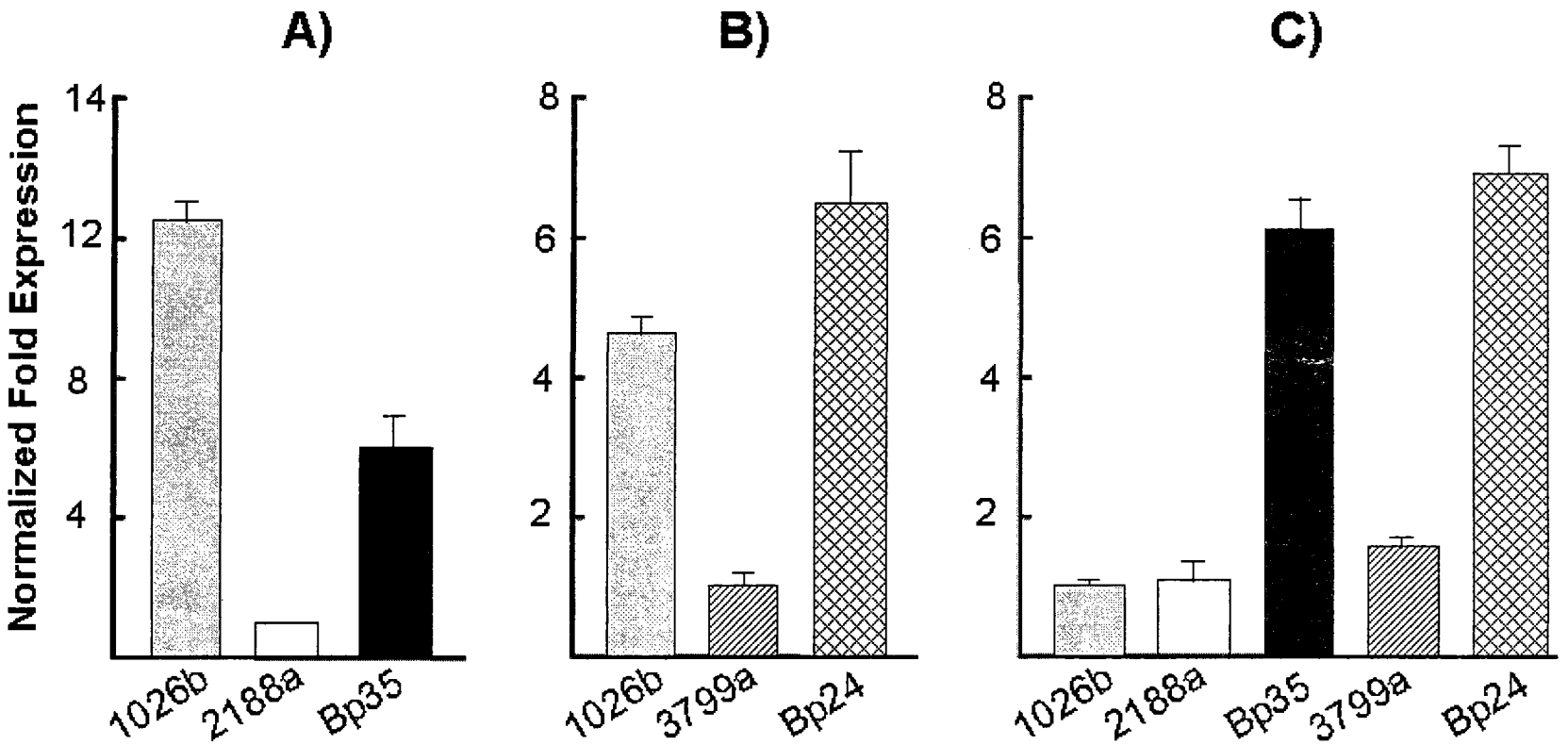


Figure 3-1 *amrB* and *amrR* transcript levels in gentamicin susceptible and resistant strains. mRNA levels in LSLB-grown late-log cultures of the indicated strains were determined with *amrB*- and *amrR*-specific primer sets. Data were normalized using the *23S rRNA* gene as the housekeeping control. *amrB* transcript levels were determined in strain 2188a and its gentamicin resistant derivative Bp35 (A) and strain 3799a and its gentamicin resistant derivative Bp24 (B). Relative quantifications were performed using 2188a and 3799a, respectively. *amrR* transcript levels relative to those found in 1026b were determined in strains 2188a, 3799a and their gentamicin resistant derivatives Bp35 and Bp24 (C)

derivatives Bp24 and Bp35 was not due to promoter-up mutations or increased expression of *amrR* or other *amrR* mutations. Rather, the data suggest that *amrAB-oprA* expression is governed by a yet unidentified transcription factor or other positive regulatory mechanism(s).

3.4.4 Strain 708a contains a large deletion on chromosome 1.

Results of PCR and *q*RT-PCR analysis were consistent with the notion that the *amrAB-oprA* operon was missing from strain 708a. Using the 1710b chromosome 1 sequence as a guide, primer sets were designed to amplify ~500 bp fragments in the *amrAB-oprA* containing region of chromosome 1. Results of this primer walking strategy identified a correct PCR product obtained with primer set 1742 and 1743 designed to amplify sequences located ~5 kb upstream of *amrR*. However, no PCR products were obtained with primers designed to sequences located up to 65 kb downstream of *oprA* (**Figure 3-2**). These data were consistent with the presence of a large (>70 kb) deletion on chromosome 1 encompassing *amrAB-oprA*. To determine the deletion boundaries, a fosmid library was constructed using 708a chromosomal DNA. By PCR amplification, several fosmids containing DNA previously located ~5 kb upstream of *amrR* were identified. Sequence analyses of both fosmid-chromosomal DNA boundaries and BLAST analyses using four *B. pseudomallei* genomes revealed the same open reading frames (ORFs) at the respective junctions, *BURPPS1710b_2037* (or its respective homolog in other genomes) and *BURPPS1710b_2160* (or its respective homolog in other genomes). A series of primers was designed to determine the sequence adjacent to the

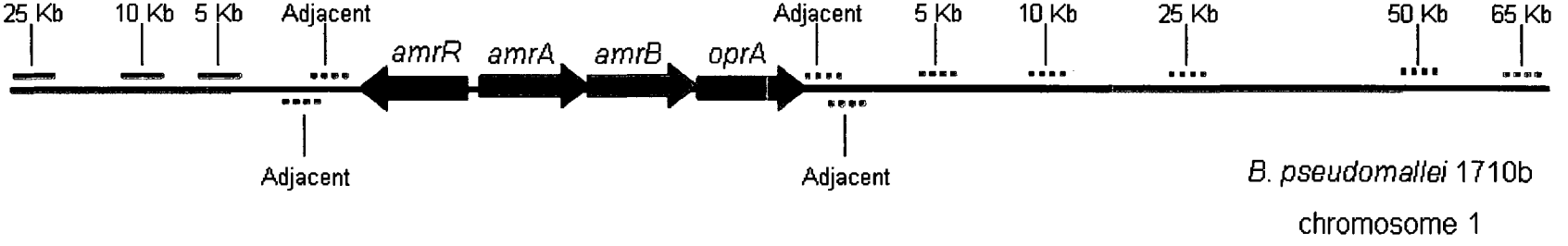


Figure 3-2 Schematic depicting primer walking strategy to identify the boundaries of the deletion containing *amrAB-oprA* in *B. pseudomallei* 708a. Using the *B. pseudomallei* 1710b sequence as a guide, primers were designed to amplify ~500 bp fragments at varying distances from the *amrAB-oprA* operon. Green solid lines indicate amplicons that could be amplified from 708a genomic DNA; red dashed lines indicate that no amplification could be achieved using 708a genomic DNA as template. Numbers above each line indicate the approximate distance of the amplicon from either upstream of *amrR* or downstream of *oprA*. The primer set 1742 and 1743 could amplify the ~500 bp fragment ~5 Kb upstream of where *amrR* should have been in strain 708a, this primer set was therefore subsequently used to screen the fosmid library for clones containing the putative deletion boundary.

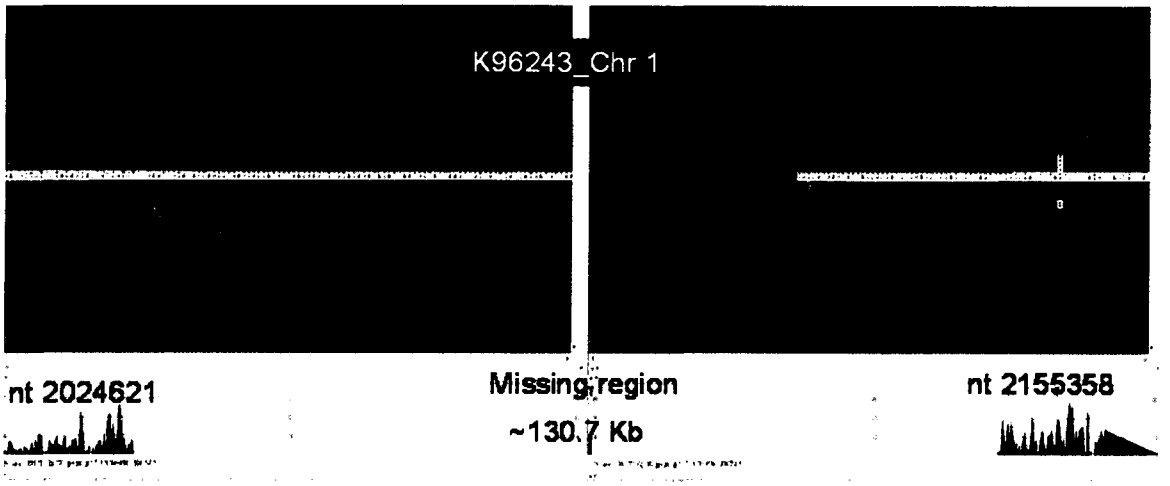
primer 1742 binding site. The sequence was aligned to that of 1710b and revealed a fusion of ORFs *BURPPS1710b_2155* and *BURPPS1710b_2054*. We interpreted this to mean that compared to 1710b, the 708a sequence was missing nucleotides 2,219,259-2,359,936 (or ~141 kb) from chromosome 1, including *amrAB-oprA*.

When compared to other strains, the extent of the deletion varied by approximately ±10 kb based on sequence from strains used as comparators. For example, when compared to K96243 the deletion is ~131 kb (**Figure 3-3 B**). The deletion was further confirmed by: i) PCR amplification using primers 1797 and 1801 and DNA sequence analysis of a 1.1 kb chromosomal DNA fragment from 708a genomic DNA containing the predicted deletion junction; and ii) whole-genome Solexa shotgun sequencing (**Figure 3-3 A**) (A. Tuanyok and P. Keim, unpublished data).

3.4.5 Genes contained within deletion present in 708a chromosome 1.

Because of the more thorough and detailed annotation of the published K96243 genome we decided to use it to assess key genes missing from *B. pseudomallei* strain 708a. According to K96243 coordinates, 708a is missing nucleotides 2,024,622 to 2,155,357 fusing the *BURPPS1710b_2155* and *BURPPS1710b_2054* equivalents *BPSL1717* and *BPSL1807* (**Figure 3-3 B**). In K96243, as well as 1710b and other *B. pseudomallei* strains, this >90 gene region not only contains *amrAB-oprA* but several other genes that may be pertinent to this bacterium's physiology and pathogenesis (**Table 3-4**). First, this deleted region also contains the 13 gene malleobactin biosynthetic gene cluster and its extracytoplasmic sigma factor MbaS defined by *BPSL1774-mbaF-fmtA-mbaA-mbaI-mbaJ-mbaE-BPSL1781-BPSL1782-BPSL1783-BPSL1784-BPSL1785-BPSL1786-mbaS* (1). Malleobactin is a hydroxamate siderophore that is analogous to

A)



B)

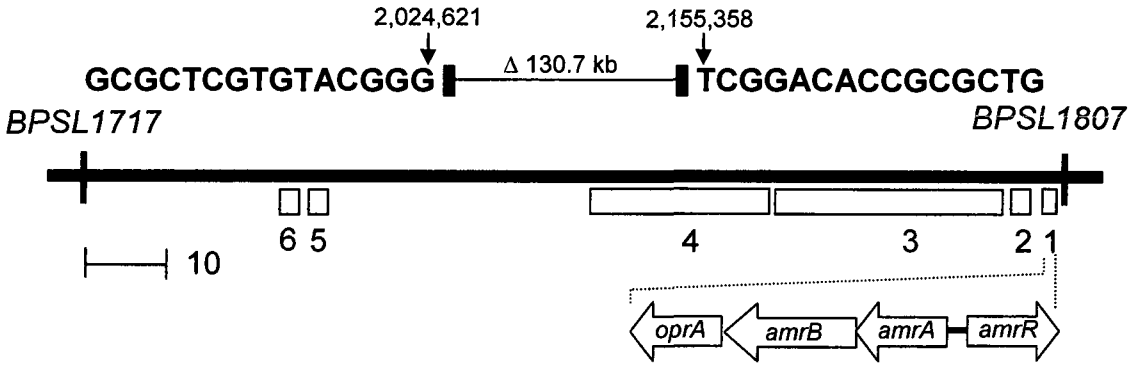


Figure 3-3 Extent of chromosome 1 deletion in strain 708a compared to K96243. **(A)** Scaffolding of the 708a sequence obtained from Solexa whole-genome shotgun sequencing against the *B. pseudomallei* K96243 published genome sequence revealed strain 708a contained a deletion of ~130.7 Kb. **(B)** Schematic depicting the 708a deletion. 708a contains a deletion fusing the bold sequences of *BPSL1717* and *BPSL1807*, respectively. Some notable genes and gene clusters present in K96243 but missing from 708a are: 1) *amrR-amrAB-oprA*; 2) a three gene operon (*BPSL1801-BPSL1800-BPSL1799*) encoding a putative type-1 fimbrial protein along with its outer membrane usher protein and chaperone; 3) the 13 gene malleobactin biosynthetic gene cluster and its extracytoplasmic sigma factor MbaS defined by *BPSL1774-mbaF-fmtA-mbaA-mbaI-mbaJ-mbaE-BPSL1781-BPSL1782-BPSL1783-BPSL1784-BPSL1785-BPSL1786-mbaS*; 4) a cluster of 18 genes (*BPSL1755-BPSL1773*) encoding a putative aerobic (or late cobalt insertion) vitamin B12 biosynthetic pathway with an embedded gene (*BPSL1763*) encoding a putative exported chitinase; 5) *arcD* (*BPSL1742*) and *arcABC* (*BPSL1743-BPSL1744-BPSL1745*) coding for the arginine deiminase pathway; and 6) a two gene cluster (*BPSL1732-BPSL1731*) coding for a putative methyl-accepting chemotaxis citrate transducer and chemotaxis protein CheW2, respectively. Strain 1710b contains an additional 10 kb of DNA in this region.

Table 3-4 K96243 gene equivalents missing from 708a chromosome 1. [1]Annotation of *BPSL1774* (*mbaF*) through *BPSL1787* (*mbaS*) according to Alice et al. (1)

Locus Tag or Gene	Putative or known function
BPSL1717	Hypothetical protein
BPSL1718	Hypothetical protein
BPSL1719	Putative kinase
BPSL1720	Putative argininosuccinate lyase
BPSL1721	Putative argininosuccinate synthase
BPSL1722	Putative formyl transferase
BPSL1723	Hypothetical protein
BPSL1724	Putative histidinol-phosphate aminotransferase
BPSL1725	Hypothetical protein
BPSL1726	Hypothetical protein
BPSL1727	Putative non-ribosomal peptide synthase (thioesterase domain)
BPSL1727	Putative non-ribosomal peptide synthase (thioesterase domain)
BPSL1728	Putative exported porin
BPSL1729	Putative AraC-family transcriptional regulator
BPSL1730	Putative transmembrane protein
BPSL1731	Chemotaxis protein CheW2
BPSL1732	Putative methyl-accepting chemotaxis citrate transducer
BPSL1733	Hypothetical protein
BPSL1734	Acyl-CoA synthase
BPSL1735	Putative transport system membrane protein
BPSL1736	Putative methyltransferase
BPSL1737	Putative ABC transport system, exported protein
BPSL1738	Putative ABC transport system, membrane protein
BPSL1739	Putative ABC transport system, ATP-binding protein
BPSL1740	Putative ABC transport system, membrane protein
BPSL1741	Hypothetical protein
BPSL1742	*arcD*
BPSL1743	*arcA*
BPSL1744	*arcB*
BPSL1745	*arcC*
BPSL1746	Short chain dehydrogenase
BPSL1747	Hypothetical protein
BPSL1748	Putative LysR-family transcriptional regulator
BPSL1749	Putative glutathione *S*-transferase
BPSL1750	Putative MarR-family transcriptional regulator
BPSL1751	Putative amino-acid transport-related exported protein
BPSL1752	Putative MarR-family regulatory protein
BPSL1753	Putative transport-related membrane protein
BPSL1754	Putative lipoprotein
BPSL1755	Precorrin-4 C11-methyltransferase
BPSL1756	Precorrin-6x reductase
BPSL1757	Cobalt-precorrin-6A synthase
BPSL1758	Precorrin-6Y C5,15-methyltransferase
BPSL1759	Putative oxidoreductase
BPSL1760	Precorrin-8X methylmutase
BPSL1761	Precorrin-2 methyltransferase
BPSL1762	Precorrin-3b C17-methyltransferase
BPSL1763	Putative exported chitinase

BPSL1764	Hypothetical protein
BPSL1765	Putative carboxylesterase
BPSL1766	Hypothetical protein
BPSL1767	Putative magnesium chelatase protein
BPSL1768	Cobaltochelatase
BPSL1769	Putative cobalamin biosynthesis-related protein
BPSL1770	High-affinity nickel transport protein
BPSL1771	Cobalamin biosynthesis protein CbiG
BPSL1772	Cob(I)yrinic acid a,c-diamide adenosyltransferase
BPSL1773	Cobyrinic acid A,C-diamide synthase
mbaF	Putative N^5-hydroxyornithine transformylase
fmtA	Malleobactin receptor
mbaA	Putative L-ornithine-N^5-oxygenase
mbaI	Putative non-ribosomal peptide synthase
mbaJ	Putative non-ribosomal peptide synthase
mbaE	Similar to *P. aeruginosa pvdE* (ABC transporter)
BPSL1780	Hypothetical protein
BPSL1781	Putative periplasmic iron-binding protein
BPSL1782	Putative ferric iron reductase
BPSL1783	Putative iron transport-related membrane protein
BPSL1784	Putative iron transport-related ATP-binding protein
BPSL1785	Hypothetical protein (similar to *syrP* from *Streptomyces verticillus*)
BPSL1786	Hypothetical protein (similar to *mbtH* from *Mycobacterium tuberculosis*)
mbaS	MbaS, extracytoplasmic sigma factor
BPSL1788	Pseudogene
BPSL1789	Short chain dehydrogenase
BPSL1790	Putative zinc-binding dehydrogenase
BPSL1791	Hypothetical protein
BPSL1792	Hypothetical protein
BPSL1793	Putative sugar-binding exported protein
BPSL1794	Putative AraC-family transcriptional regulator
BPSL1795	Hypothetical protein
BPSL1796	Hypothetical protein
BPSL1797	Putative ABC transport system, membrane protein
BPSL1798	Hypothetical protein
BPSL1799	Putative fimbrial chaperone
BPSL1800	Putative outer membrane usher protein precursor
BPSL1801	Putative type-1 fimbrial protein
BPSL1802	OprA multidrug efflux outer membrane channel protein
BPSL1803	AmrB multidrug efflux system transporter protein
BPSL1804	AmrA multidrug efflux system membrane fusion protein
BPSL1805	AmrR TetR family regulatory protein
BPSL1806	Subfamily M23B unassigned peptidase
BPSL1807	Putative amino acid transport system, membrane protein

the same genes in *Pseudomonas aeruginosa* pyoverdine (37) and *B. cepacia* ornibactin(31). Pyoverdine is essential for infection and virulence of *P. aeruginosa*, as assessed in several different experimental models (36), along with biofilm formation (3). Similarly, *B. cepacia* mutants defective in ornibactin synthesis showed significantly

reduced virulence (31). However, in the case of 708a, despite missing the entire malleobactin biosynthetic gene cluster and exhibiting overall greatly reduced siderophore synthesis (as assessed by growth on Chrome azurol S plates) (1, 29)(data not shown), the 708a strain was still able to cause severe illness in the infected human from which it was isolated (**Table 3-2**). Moreover, strain 708a was also fully virulent in our acute inhalational challenge model in mice (**Figure 3-4**). Thus, it is possible that malleobactin may not play the same crucial role in infection and virulence that the *P. aeruginosa*

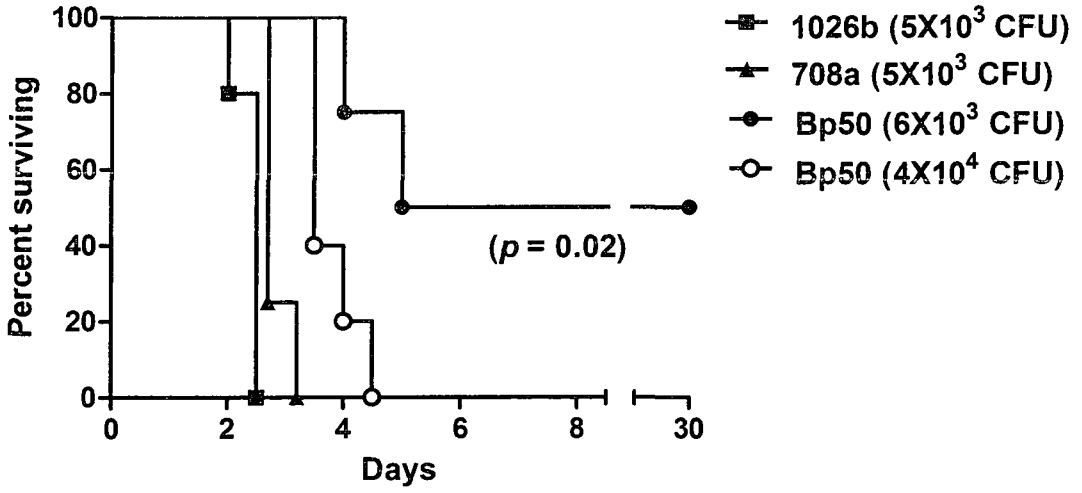


Figure 3-4 Strain 708a is fully virulent in an acute murine melioidosis infection model. BALB/c mice (n = 4-5) were infected intranasally with the indicated strains and colony forming units. Bp 50 is an isogenetic Δ(*amrRAB-oprA*) derivative of strain 1026b.

pyoverdine siderophore does. Alternatively, *B. pseudomallei* is known to synthesize other iron transport systems, including a pyocheline siderophore and heme-hemin receptor and transporter (1, 34), and thus 708a may utilize these alternative pathways for iron transport.

Second, immediately adjacent to the malleobactin biosynthetic genes is a cluster of 18 genes (*BPSL1755-BPSL1773*) encoding a putative aerobic (or late cobalt insertion)

vitamin B12 biosynthetic pathway (40). Vitamin B12 is a known cofactor for numerous enzymes mediating methylation, reduction, and intramolecular rearrangements. Why this pathway is dispensable for growth in 708a is not known. However, some bacteria are known to possess an alternative anaerobic (or early cobalt insertion) pathway (40). Third, the deletion in 708a encompasses the genes *arcD* (*BPSL1742*) and *arcABC* (*BPSL1743-BPSL1745*) coding for the arginine deiminase pathway. In *P. aeruginosa*, this pathway provides for ATP synthesis under anaerobic conditions in the absence of exogenous electron acceptors provided that arginine is present in the growth medium (35). In this context it is worthy of note that 708a was isolated from a splenic abscess and abscesses are generally considered to provide a mixed aerobic-anaerobic environment (5, 6). If 708a was truly able to grow under anaerobic conditions, then 708a must be capable of utilizing alternative pathways for energy generation under anaerobic conditions. This alternate pathway would presumably require nitrate as *B. pseudomallei* was shown to be capable of growing anaerobically only in the presence of arginine and nitrate (43). Fourth, other noteworthy genes covered by the deletion include i) a three gene operon (*BPSL1801-BPSL1799*) encoding a putative type-1 fimbrial protein along with its outer membrane usher protein and chaperone; ii) a two gene cluster (*BPSL1732-BPSL1731*) coding for a putative methyl-accepting chemotaxis citrate transducer and chemotaxis protein CheW2, respectively; and iii) a putative exported chitinase (*BPSL1763*).

In summary, these findings provide insight into the physiology and pathogenesis of *B. pseudomallei*. However, because 708a grows normally in rich and minimal laboratory media under aerobic conditions, is fully virulent in an acute murine

melioidosis model and caused human melioidosis, the genes affected by the deletion must be dispensable at least under the *in vitro* and *in vivo* conditions encountered during laboratory studies and splenic abscess disease during human infection caused by lone presence of 708a. This scenario is likely as simultaneous infection with more than one strain is uncommon in human melioidosis (19).

3.5 Conclusions

The clinical diagnosis of *Burkholderia pseudomallei* still relies on culture which is most commonly performed using selective Ashdowns agar whose main selective ingredient is gentamicin. *B. pseudomallei* grows on this medium because of its intrinsic resistance to aminoglycosides mediated by the AmrAB-OprA efflux pump. At least 1 in 1,000 clinical isolates in northeastern Thailand are susceptible to aminoglycosides and such isolates are obviously missed by using Ashdown's diagnostic agar. Our results confirm that the aminoglycoside and macrolide susceptibility of rare clinical isolates is indeed due to reduced or lack of expression of the *amrAB-oprA* efflux pump operon, as previously suggested but not proven (30). Even though BpeAB-OprB was previously implicated to contribute to aminoglycoside and macrolide resistance in strain KHW (7), we now know that this pump does not confer aminoglycoside resistance in 1026b (T. Mima and H. Schweizer, unpublished observations), a strain isolated in the same hospital as 708a. BpeAB-OprB is only expressed at very low levels in wild-type strains which may explain the low levels of erythromycin resistance observed in 708a, 2188a and 3799a in the absence of AmrAB-OprA. This notion is supported by the observation that all strains analyzed in this study exhibit clindamycin resistance. Clindamycin is a substrate of BpeAB-OprB but not AmrAB-OprA (T. Mima and H. Schweizer,

unpublished data). As expected, *q*RT-PCR analyses showed only low-level *bpeAB-oprB* expression in these strains (data not shown). Though strain 708a contains a large deletion encompassing several gene clusters encoding potential virulence factors and genes required for growth under anaerobic conditions, these genes may either be dispensable for *in vitro* and *in vivo* growth or this strain compensates for them by expressing similar functions from another set of genes. The latter notion may be supported by the observation that the genetically engineered 1026b AmrAB-OprA mutant derivative Bp50 shows reduced virulence in the murine melioidosis model whereas 708a missing these genes is as virulent as 1026b (**Figure 3-4**). We do not know the factors, if any, that led to selection of strains missing or lacking expression of AmrAB-OprA. Further experiments aimed at addressing some of these issues at the molecular level are facilitated by availability of the complete 708a sequence and tools that allow genetic manipulation of this strain. Lastly, because 708a is fully virulent in the murine melioidosis model, yet very susceptible to aminoglycosides, this strain may be a natural candidate for genetic manipulation experiments that use Select Agent compliant antibiotics for selection, such as gentamicin (10), kanamycin (10), spectinomycin/streptomycin (26) and nourseothricin (21) selection markers, and validates the use of laboratory-constructed Δ(*amrAB-oprA*) mutants in such experiments (10, 24).

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CHAPTER 4

RND EFFLUX PUMP EXPRESSION AND CORRELATION TO ANTIBIOTIC RESISTANCE PROFILE IN CLINICAL AND ENVIRONMENTAL *Burkholderia pseudomallei* ISOLATES FROM THAILAND

4.1 Abstract

Burkholderia pseudomallei, the etiologic agent of melioidosis, is a category B select agent. Due to *B. pseudomallei's* intrinsic antibiotic resistance, treatment of melioidosis is costly and extensive. Expression of at least one efflux pump, AmrAB-OprA, significantly contributes to the antibiotic resistant nature of *B. pseudomallei* in most strains, but not much is known about the expression of at least six other RND efflux pumps and their potential contribution to antibiotic resistance. A survey of efflux pump expression in clinical and environmental strains from Thailand was conducted to assess patterns of pump expression and potential correlation to antibiotic resistance.

Quantitative real-time PCR (*q*RT-PCR) was used to analyze the expression of seven *B. pseudomallei* efflux pumps (characterized: *amrB, bpeB,* and *bpeF* putative: *bpeH, BPSL0309, BPSL1267*, and *BPSL1567*) in 60 isolates (30 clinical and 30

environmental) from Thailand. MICs of nine antibiotics (gentamicin, erythromycin, ceftazidime, co-trimoxazole, doxcycline, co-amoxiclav, chloramphenicol, levofloxacin, and trimthoprim) representing most major classes of clinically significant antimicrobials were determined by E-Test. Welch's *t*-test was used to determine whether any of the seven efflux pumps was differentially expressed in clinical and environmental strains. *bpeH* had significantly higher expression in the clinical strains as compared to the environmental strains. This pump has yet to be characterized; however, its expression in clinical isolates may indicate its importance for survival in this niche or its possible contribution to multi-drug resistance. Linear regression and ANOVA were used to elucidate correlations between MIC for any of the nine antimicrobials tested and expression levels of each of the seven pumps studied. These statistical tests identified a direct correlation between resistance to gentamicin and expression of both *BPSL0309* and *bpeH.* Additionally inverse relationships where identified between the expression of several pumps and resistance to co-trimoxazole, gentamicin, ceftazidime, co-amoxiclav, and doxycycline. The inverse relationships between pump expression and MIC were surprising findings but may provide some insight into possible co-regulation of RND efflux pumps and other resistance determinants in *B. pseudomallei.* Further characterization of efflux pumps in this organism may contribute to the development of more effective chemotherapies by aiding in the discovery of new drugs that are poor substrates of these pumps or efflux pump inhibitors for use in combination with existing antibiotics.

4.2 Introduction

Burkholderia pseudomallei is the etiological agent of melioidosis. This disease is endemic to tropical regions and has been recognized by the Centers for Disease Control (CDC) as a potential bio-threat, hence it's classification as a category B select agent (10, 35). Treatment of melioidosis is difficult due to the high level of intrinsic resistance of *B. pseudomallei* to aminoglycosides, macrolides, and third generation cephalosporins, among others (16, 34). Bioinformatic analysis of the *B. pseudomallei* genome revealed the presence of at least 10 RND efflux pumps (14). Pumps belonging to the Resistance-Nodulation-Cell Division (RND) family are the most relevant type of efflux pump in Gram negative bacteria. This is attributed to their unique tripartite architecture which allows them to extrude substrates through both the cytoplasmic and outer membranes directly to the extracellular space (22).

Of the ten putative RND efflux systems in *B. pseudomallei*, only 7 are likely candidates for multidrug resistance determinates (**Figure 4-1**). Based on sequence homology, the remaining three have more likely roles in general protein secretion, metal ion transport, and cation efflux (21). Three of these RND operons have been characterized (AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC). Aminoglycosides and macrolides have been named as substrates for both AmrAB-OprA and BpeAB-OprB (7, 28). Using a surrogate *Pseudomonas aeruginosa* strain, chloramphenicol and trimethoprim were identified as substrates for BpeEF-OprC (20). Using a different strain background than the original investigators (1026b as opposed to KHW), our results indicate that aminoglycosides are not substrates for BpeAB-OprB. Instead, this pump's substrates include quinolones, macrolides, lincosamides and tetracycline (T. Mima and H.P. Schweizer, unpublished results). Nothing is known about the four remaining

putative pumps (*BPSL0307- BPSL0308- BPSL0309, BPSL1268- BPSL1267, BPSL1568- BPSL1567- BPSL1566,* and *BPSS1118- BPSS1119- BPSS1120* [aka *bpeGH-oprD*]). A recent study found that these pumps were widely expressed in Australian clinical isolates but did not analyze the potential correlation between efflux pump expression and antibiotic resistance in those strains (21).

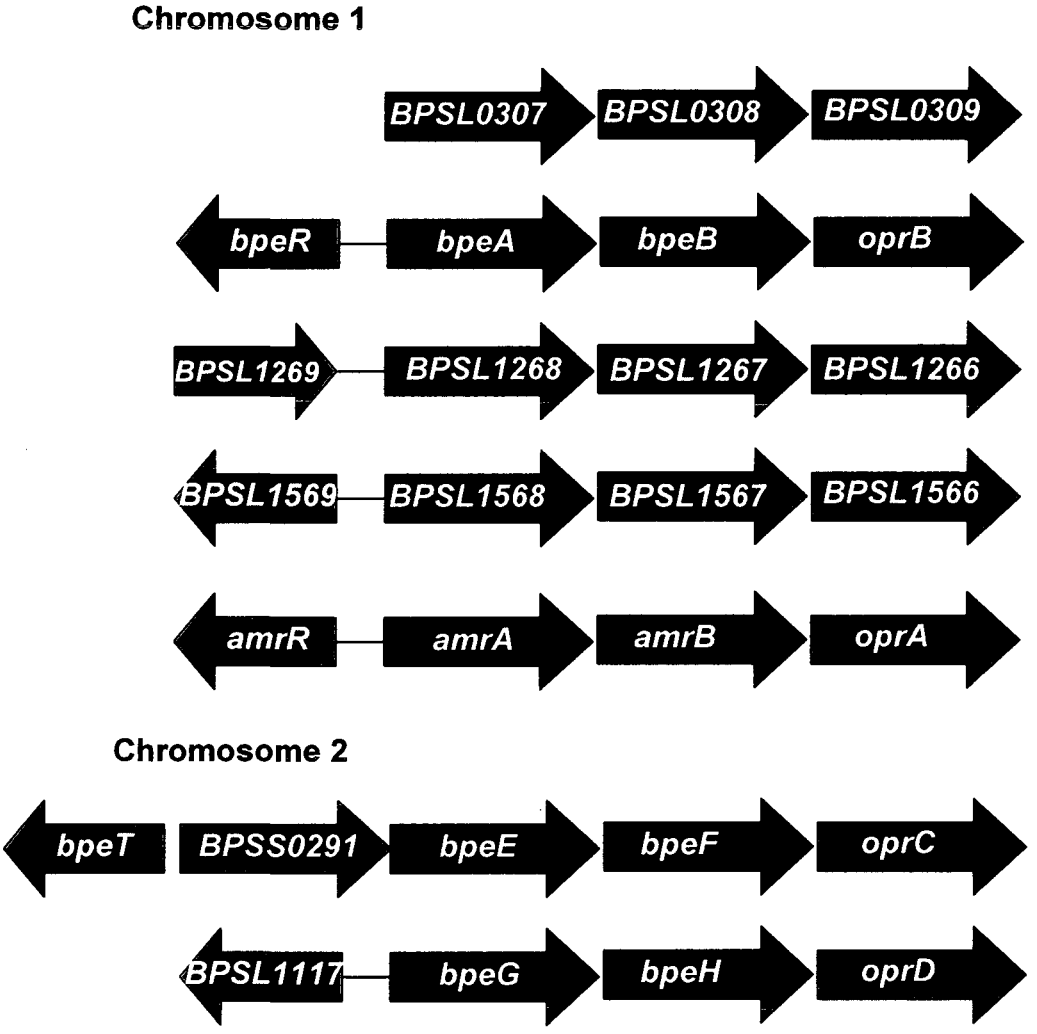

Figure 4-1 Schematic of the genetic organization of RND efflux operons in *B. pseudomallei* K96243. The gene for the membrane fusion protein is depicted in blue, RND transporter in maroon, and outer membrane protein in green. If there is a local regulator associated with the operon, it is depicted in pink. *BPSL1268-BPSL1267-BPSL1266* is predicted to have two RND transporters while *bpeEF-oprC* has a putative lipase associated with the operon

As there is extensive evidence of geographically based strain differences, this study aims to profile RND efflux pump expression in a collection of clinical and environmental strains from Thailand (8, 11, 33). Genetic distinctions have been observed between clinical and environmental isolates and, given that the physiologic role of efflux pumps is unclear, we were interested in determining whether these pumps were differentially expressed in clinical and environmental strains (27, 29, 37). Finally, in an effort to identify potential substrates for the uncharacterized pumps, we sought to correlate efflux pump expression (as measured by *q*RT-PCR) and minimum inhibitory concentration (MIC) of various antibiotics representing nearly every class of antimicrobials, especially those that are relevant to the treatment of melioidosis.

4.3 Materials and methods

4.3.1 Bacterial strains and culture conditions.

Sixty *B. pseudomallei* isolates were obtained from the culture collection at the Wellcome Unit of Mahidol University's Faculty of Tropical Medicine in Bangkok, Thailand. These isolates, which are identified in **Table 4-1,** included 30 clinical isolates and 30 environmental isolates. Unless otherwise indicated, bacteria were cultured in Luria-Bertani (LB) medium (31) with aeration at 37° C. Muller-Hinton II agar (MHA II; Difco, Detroit, Mich.) was used with the E-Test for antimicrobial susceptibility testing.

4.3.2 RNA extraction and cDNA sythesis

RNA extractions were carried out using the Qiagen RNeasy kit (Cybeles, Bangkok, Thailand). One milliliter of late log phase (A_{600nm} ~0.7) culture was harvested by centrifugation at 13,000 rpm, the supernatant was discarded and the pellet was frozen at -80° C to facilitate lysis. RNA isolation was performed following the manufacturer's

Table 4-1 Bacterial strains used for RND efflux pump expression analysis

Isolate	Isolation site	Isolate ID
1	blood	2613a
2	pus	2614a
3	pus	2617a
4	pus	2618a
5	blood	2625a
6	pus	2637a
7	pus	2640a
8	blood	2650a
9	blood	2660a
10	blood	2661a
11	blood	2665a
12	blood	2667a
13	blood	2668a
14	tracheal suction	2670a
15	blood	2671a
16	blood	2673a
17	blood	2674a
18	sputum	2677a
19	blood	2682a
20	pus	2685a
21	blood	2689b
22	blood	2692a
23	sputum	2694a
24	blood	2698a
25	blood	2704a
26	pus	2708a
27	pus	2717a
28	tracheal suction	2719a
29	blood	2764b
30	pus	2769a
31	environmental	E0008
32	environmental	E0016
33	environmental	E0021
34	environmental	E0024
35	environmental	E0031
36	environmental	E0034
37	environmental	E0037
38	environmental	E0181
39	environmental	E0183
40	environmental	E0235
41	environmental	E0237
42	environmental	E0241
43	environmental	E0279
44	environmental	E0342
45	environmental	E0345
46	environmental	E0350
47	environmental	E0356
48	environmental	E0366
49	environmental	E0371
50	environmental	E0372
51	environmental	E0377
52	environmental	E0378
53	environmental	E0380
54	environmental	E0383
55	environmental	E0384
56	environmental	E0386
57	environmental	E0393
58	environmental	E0394
59	environmental	E0396
60	environmental	E0411

instructions. RNA was treated with RQ1 RNase-free DNase from Promega (Bio-Active, Bangkok, Thailand) prior to cDNA synthesis. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE). cDNA synthesis was carried out using 1 μg DNase treated RNA and the SuperScript III First-Strand Synthesis Supermix for *q*RT-PCR from Invitrogen (Gibthai Company, Bangkok, Thailand) following the manufacturer's instructions.

4.3.3 Quantitative real-time PCR and expression analysis

Quantitative real-time PCR (*q*RT-PCR) was performed using the SYBR GreenER *q*RT-PCR master mix (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Primers used for this study, shown in **Table 4-2**, were the same as used by Kumar et al. (21). cDNA samples were diluted 1:10 in DNase-free water before use as template in *q*RT-PCR. The *23s* rRNA gene was used as an internal control. Each isolate was run in triplicate with each primer set (technical replicates).

Table 4-2 Primers used for *q*RT-PCR

Target gene	Primer name	Sequence (5'→3')
23s rRNA	1516	gta gac ccg aaa cca ggt ga
	1517	cac ccc tat cca cag ctc at
amrB	1398	gtc agc acg ttg atc gag aa
	1399	cgc tgt gat gtt cct ctt ca
bpeB	1526	ggc ctc gac aac ttc ctg ta
	1527	ttc ttc tgc acc tga acc tg
bpeF	1524	tcc gag tat ccg gaa gtc gt
	1525	gtc ctc gac acc gtt gat ct
bpeH	1528	cat tca acg aga tgg tcg tg
	1529	cgc gct cga gtt gta gtt ct
BPSL0309	1552	gtg ggc tgg gtc tac gaa ta
	1553	agc tcg aac ttc agg aac ca
BPSL1267	1556	gaa cca gct gtt cct gat cc
	1557	cgg atg gat gta gct ctc gt
BPSL1567	1554	aat aca cgc gct cga tct tc
	1555	ctc ggg cag cat gtg ata g

4.3.4 Antibiotic susceptibility testing

Minimum inhibitory concentrations (MIC) were determined using E-test strips (AB Biodisk, Solna, Sweden) following the protocols provided by the supplier.

4.3.5 Statistical analysis

Comparison of efflux pump expression in clinical and environmental isolates was accomplished by comparing mean relative fold expression values for each group (in regard to one target gene at a time) using Welch's *t*-test in Excel 2003 for Windows. The association between MIC and pump expression level was assessed using two separate statistical methods: ANOVA and linear regression; both were done in Excel 2003 for Windows. In ANOVA, MIC was considered an independent, categorical variable (x) and mean relative fold expression was the dependent variable (y), whereas using linear regression, MIC is considered a continuous independent variable. Relationships suggested by a significant *p*-value in the ANOVA analysis were further elucidated using both the LSD approach (conservative) as well as the Waller-Duncan (liberal) method using SAS 9.2 for Windows. Linear regression analysis was performed in Excel 2003 for Windows. For all statistical analysis, only *p*–values <0.05 were considered significant. Similar statistical analysis performed by Ruzin et al. to determine correlations between expression of efflux pump regulators and resistance to tigecycline (30).

4.4 Results

4.4.1 Expression of RND efflux pumps differs between individual Thai isolates

The expression of seven RND efflux pumps, whose genetic arrangements are shown in **Figure 4-1**, was measured in 60 Thai isolates of *B. pseudomallei*. Expression

was measured using *q*RT-PCR and relative quantification was calculated using the Pfaffl method, a derivation of the $\Delta\Delta C_t$ method which accounts for differences in primer efficiencies of target and reference primer sets (9, 26, 32, 36). Rather than measuring expression of target genes relative to a reference strain, expression was measured relative to the strain with the lowest cycle threshold value (C_t) for each target gene (15, 18). This minimizes the bias of using a single strain as a reference strain, which was particularly important in this study because complete characterization of RND efflux has not been accomplished in any single *B. pseudomallei* strain (20, 30). While this method of calculating relative expression allows for comparisons of each pump between strains, one limitation is that expression of each pump relative to the other pumps cannot be assessed. Expression of each pump is variable across the 60 strains analyzed; this is illustrated in **Figures 4-2** through **4-8.**

4.4.2 Putative RND efflux transporter, *bpeH*, is more highly expressed in Thai clinical isolates as compared to environmental isolates.

In order to assess differences in expression of RND efflux transporters in clinical and environmental *B. pseudomallei* isolates, expression levels were compared using Welch's *t*-Test, the results of which are summarized in **Table 4-3.** Because there was a wide range of expression, relative expression levels were converted to a $\log_2$ scale to facilitate analysis. Only *p*-values <0.05 indicated a significant difference in expression. These results indicate that in Thai isolates, expression of *bpeH* is significantly higher in clinical strains than in environmental strains. This is depicted graphically in **Figure 4-9.**

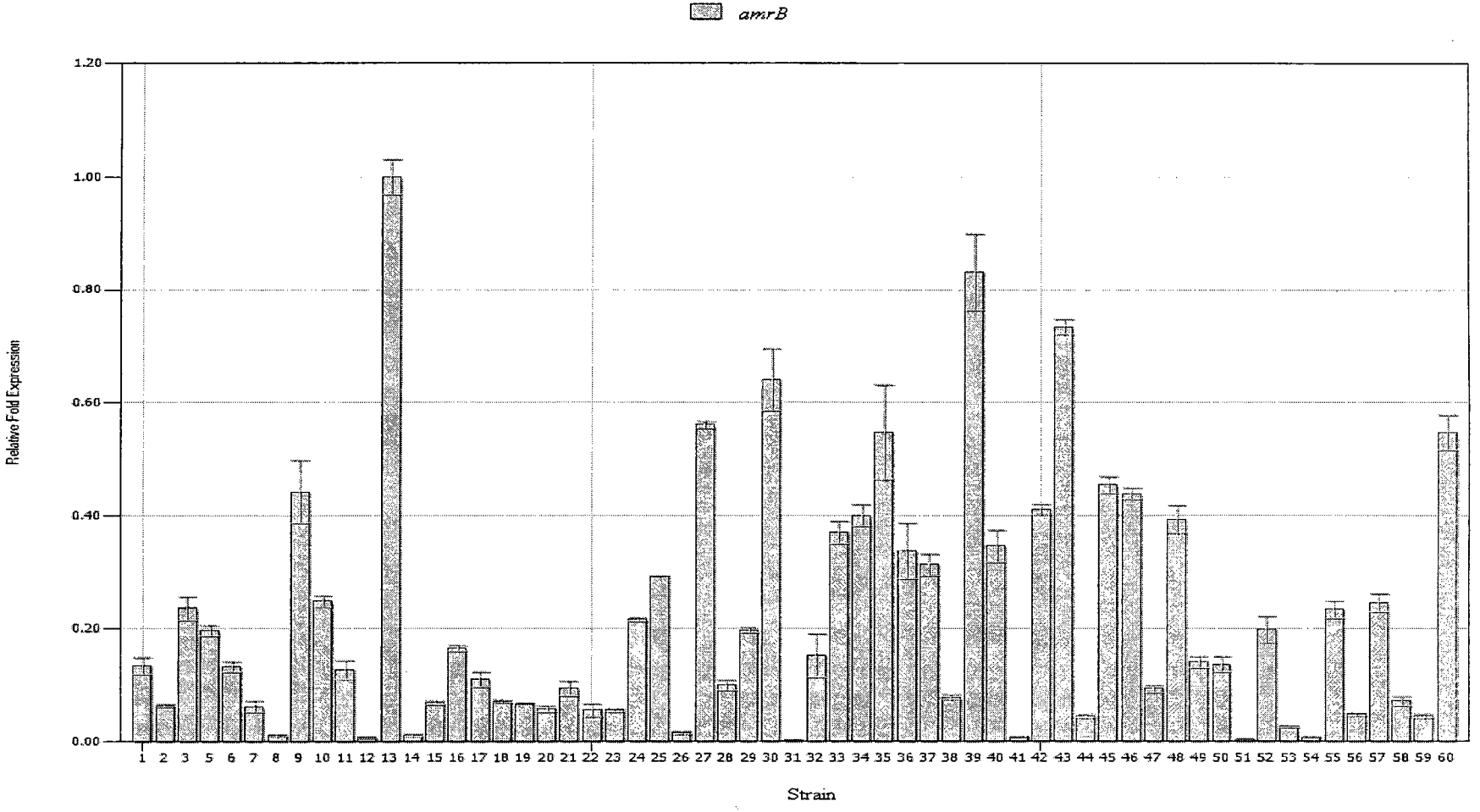

Figure 4-2 Relative expression of *amrB* in 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

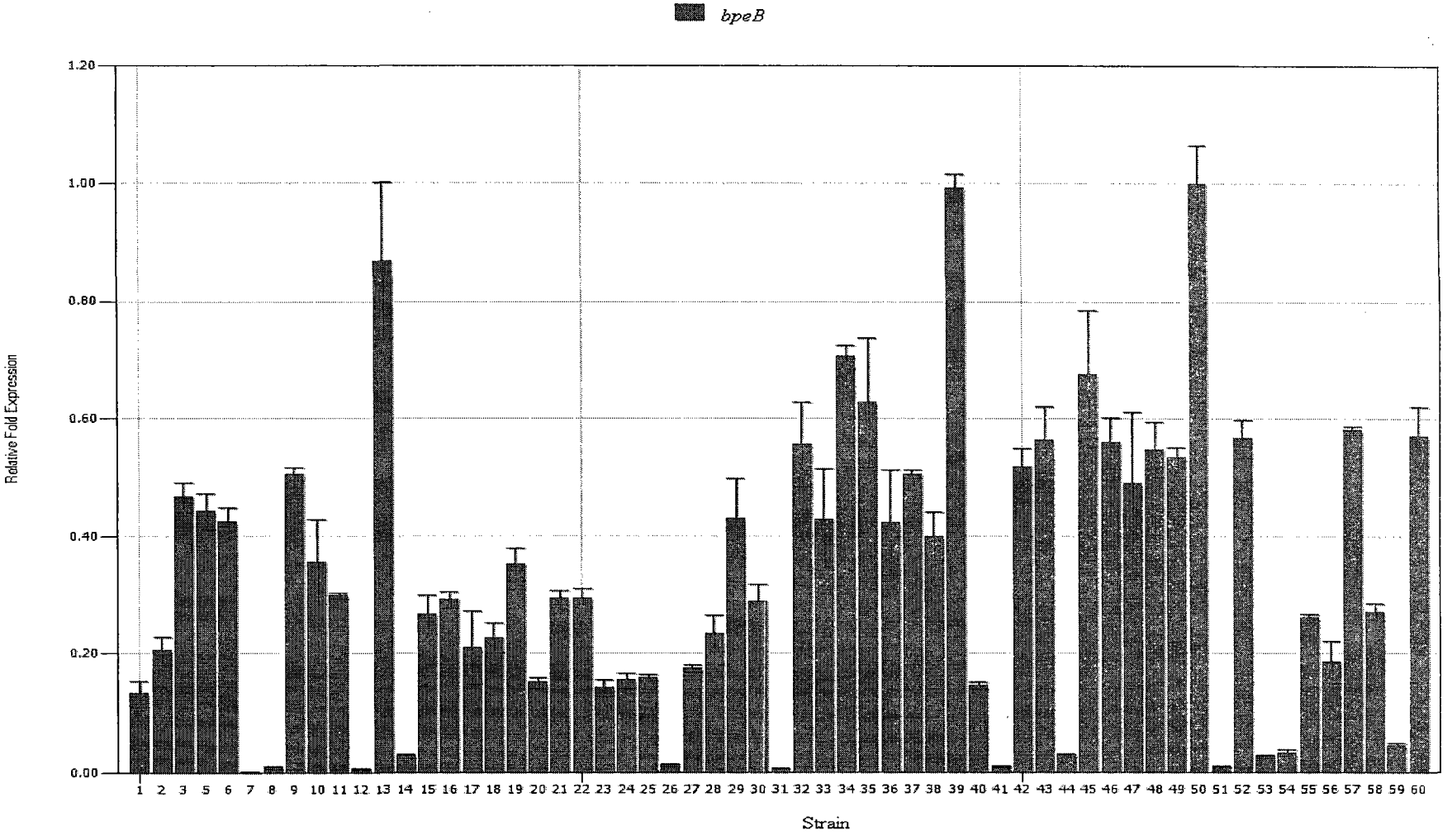

Figure 4-3 Relative expression of *bpeB in* 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

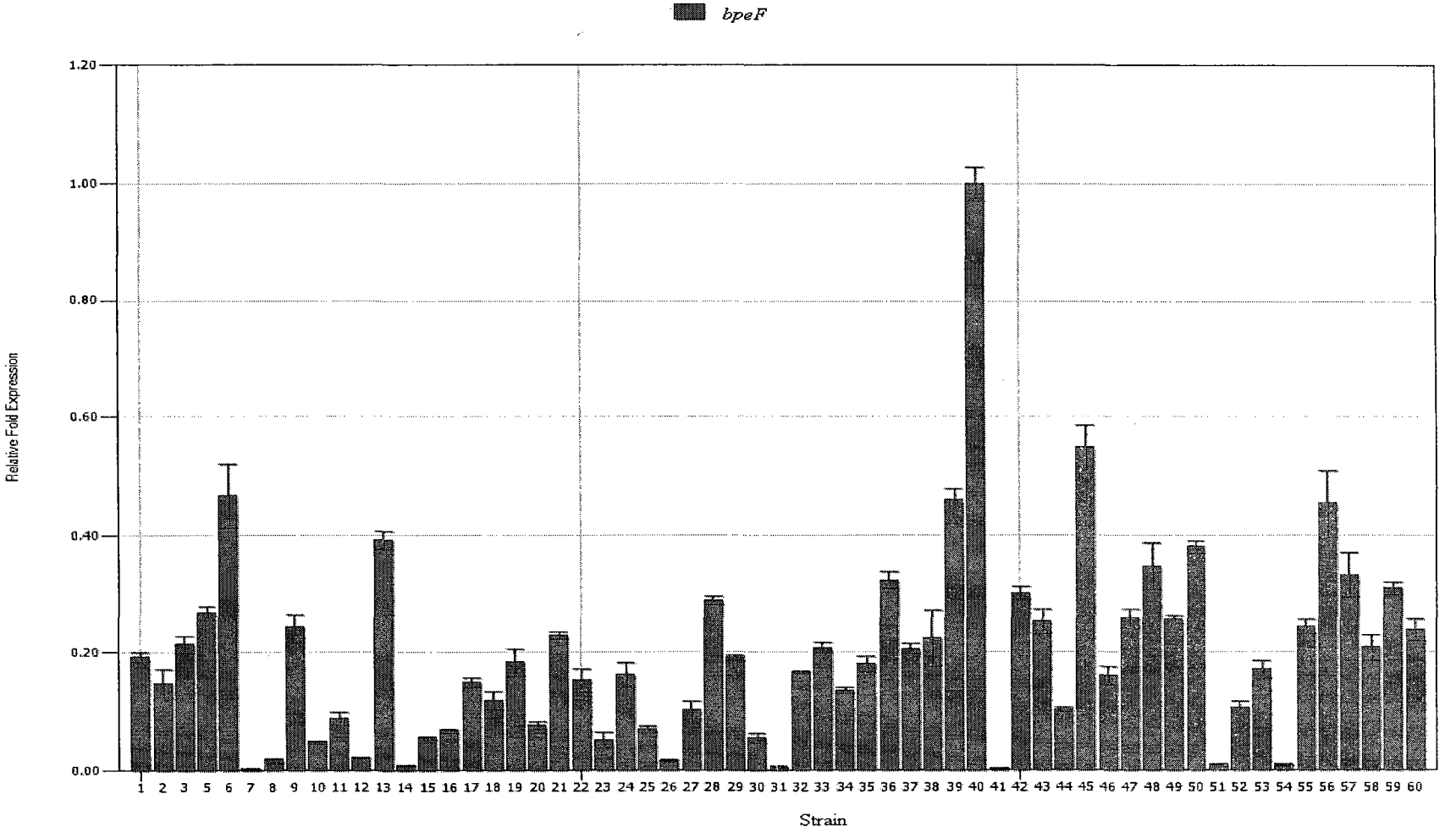

Figure 4-4 Relative expression of *bpeF in* 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

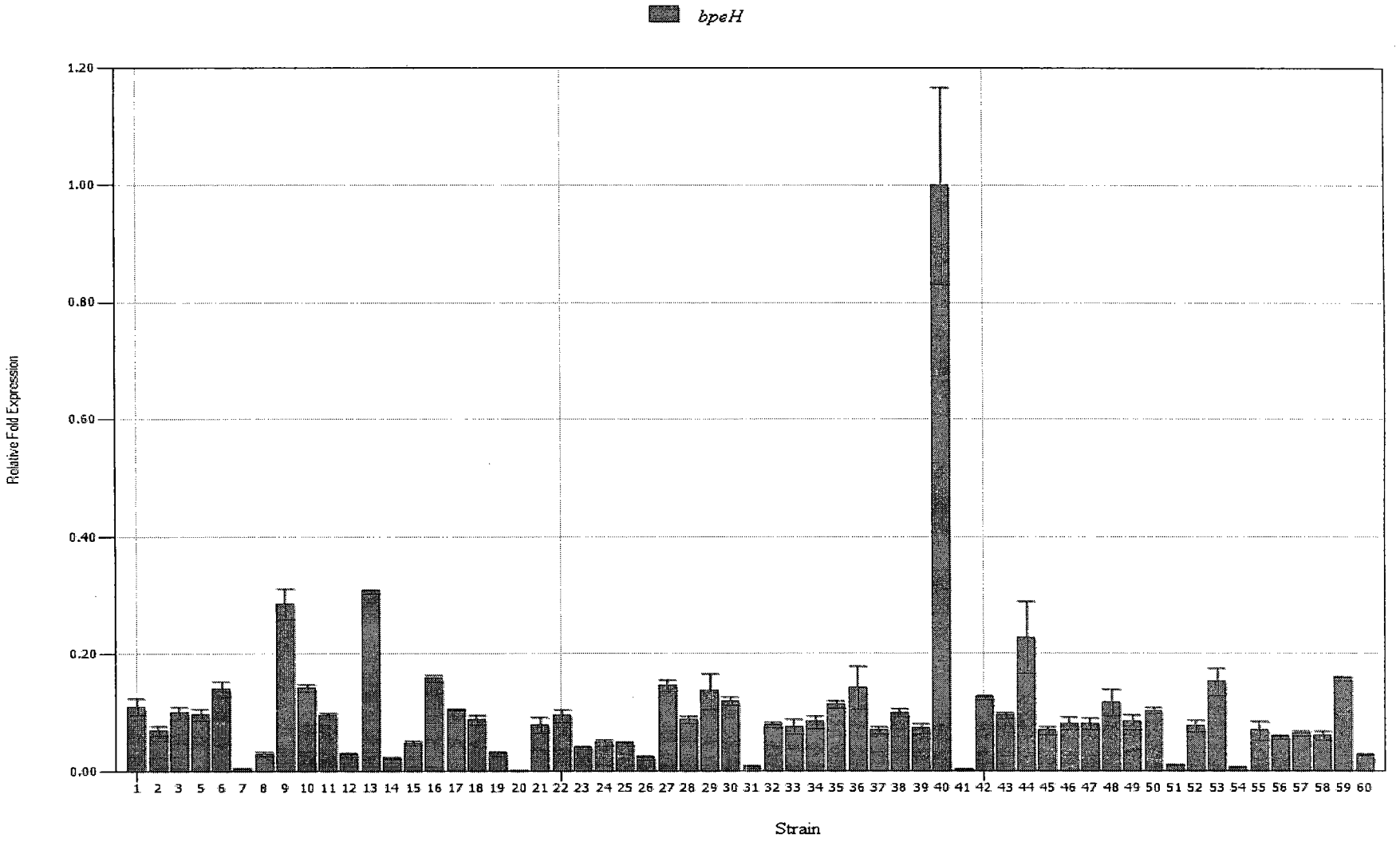

Figure 4-5 Relative expression of *bpeH in* 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

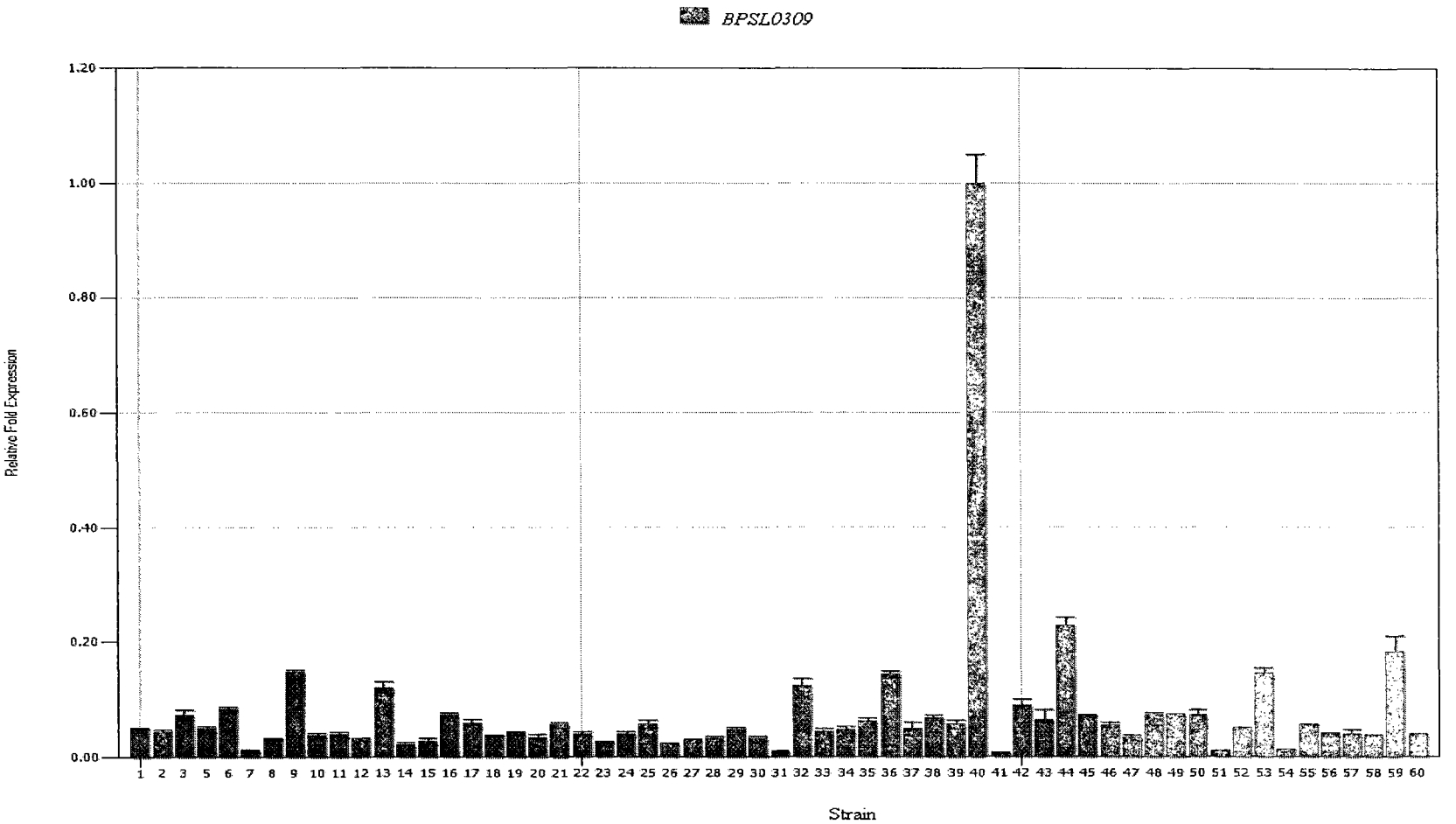

Figure 4-6 Relative expression of *BPSL0309 in* 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

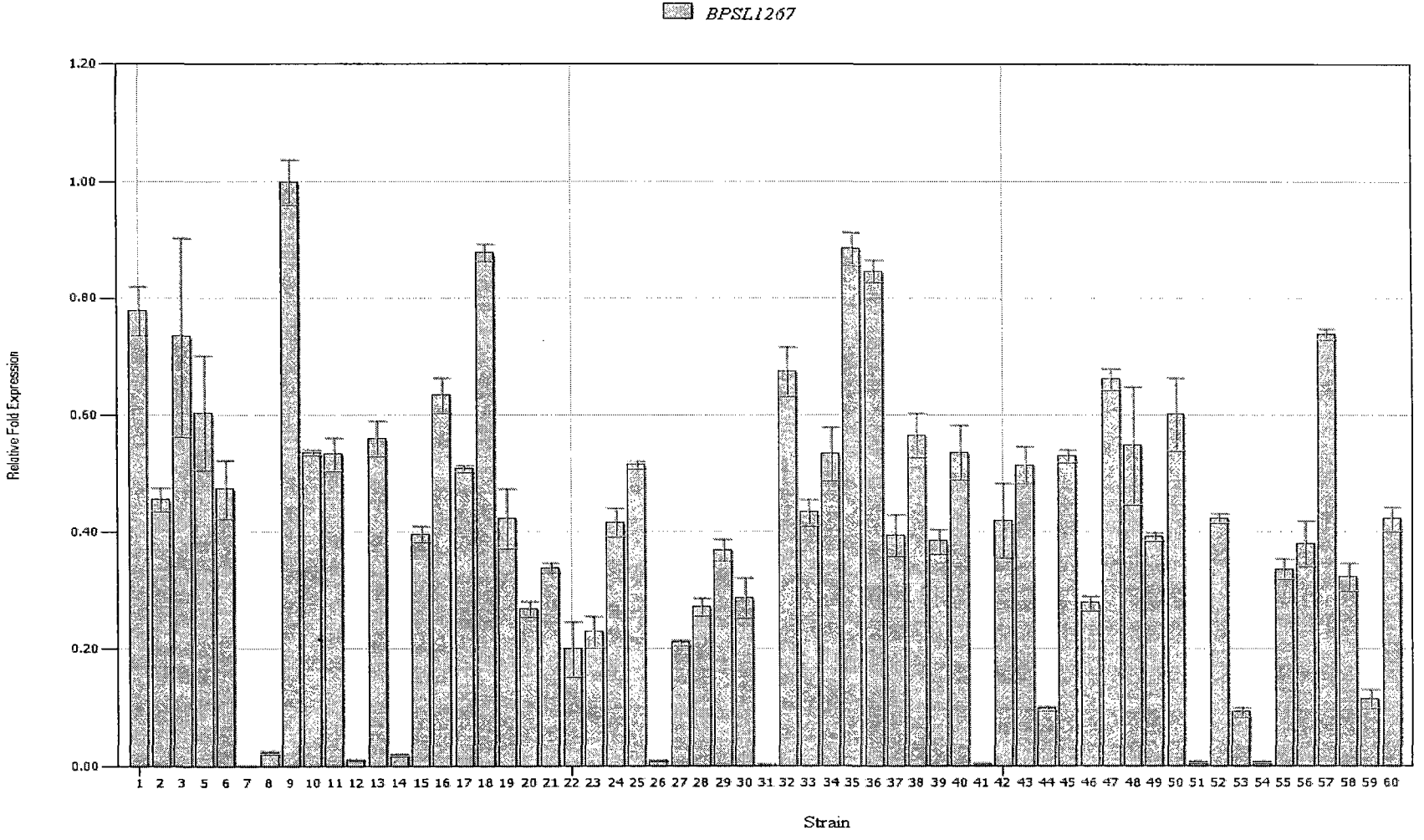

Figure 4-7 Relative expression of *BPSL1267 in* 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

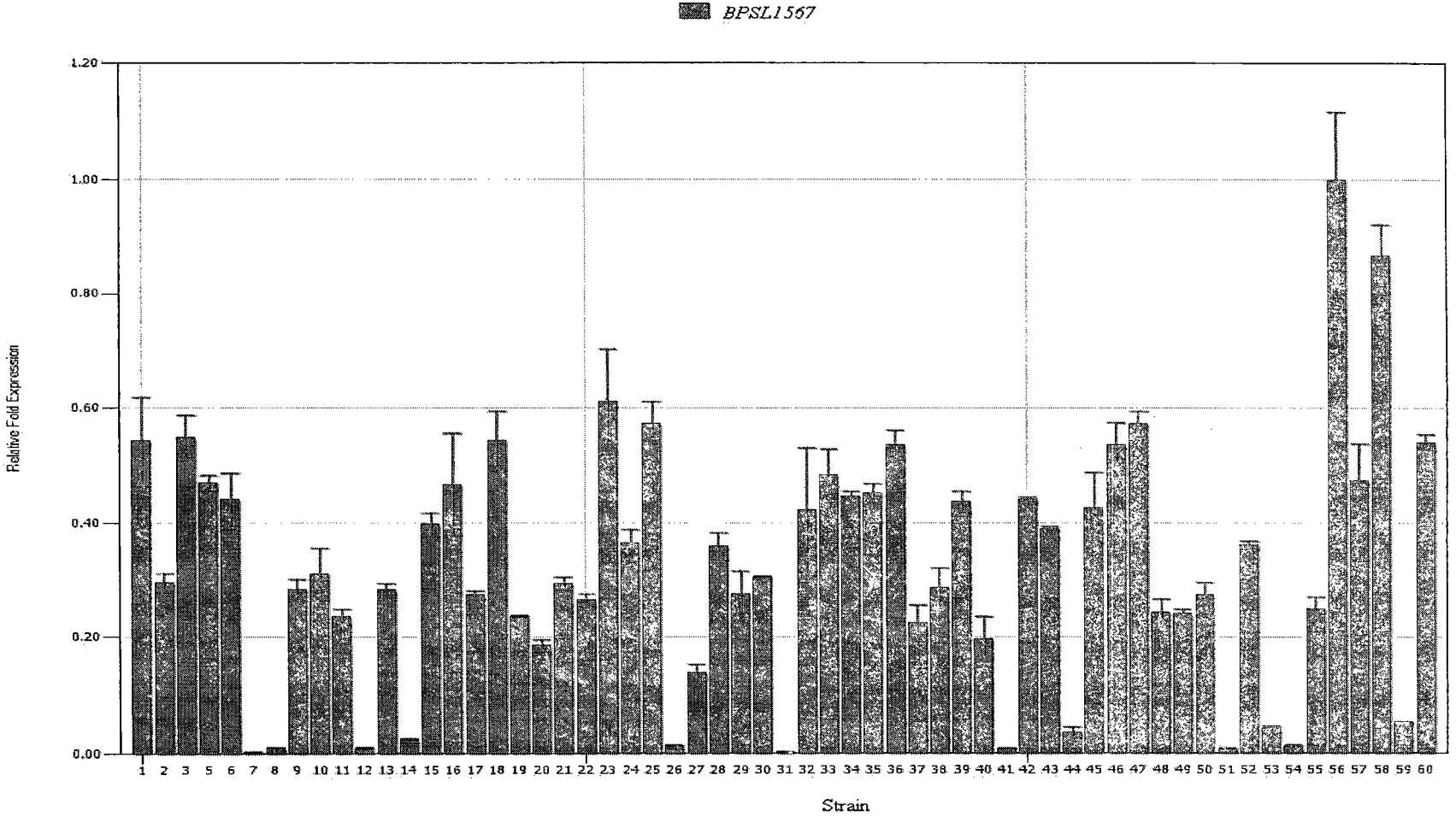

Figure 4-8 Relative expression of *BPSL1567 in* 60 *B. pseudomallei* isolates from Thailand. Isolates 1-30 are from clinical specimens, 31-60 are environmental. Error bars represent variation between technical replicates for each isolate.

Table 4-3 Summary of Welch's *t*-test comparing RND pump expression in clinical and environmental isolates. Significant *p*-values (<0.05) are boldfaced.

	amrB	*bpeB*	*bpeF*	*bpeH*	*BPSL0309*	*BPSL1567*	*BPSL1267*
***p*-value from Welch's *t*-Test**	0.15	0.39	0.59	**0.04**	0.16	0.06	0.08
Mean relative expression in clinical isolates (log_2)	0.85	1.35	0.62	0.12	-0.46	2.01	1.67
Mean relative expression in environmental isolates (log_2)	0.02	0.86	0.32	-0.93	-1.17	0.85	0.60

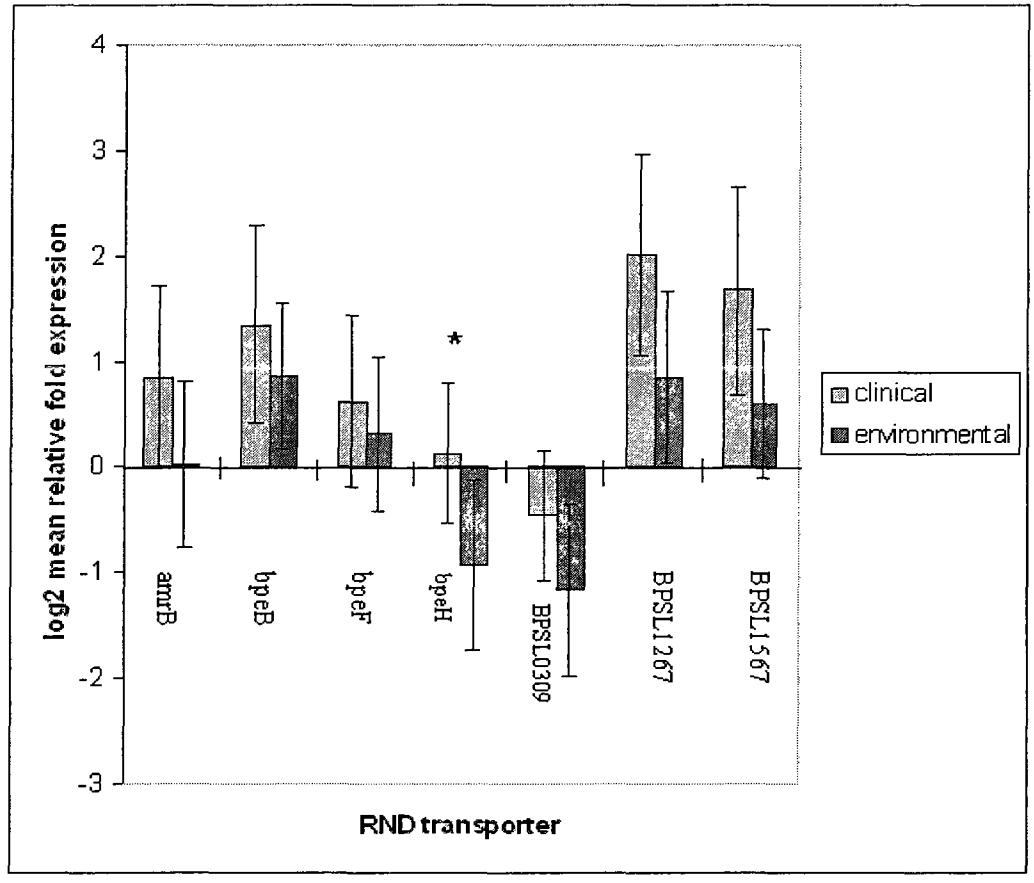

Figure 4-9 Log_2 transformed mean expression of RND efflux transporters in clinical and environmental strains of *B. pseudomallei*. (*) indicates significantly different expression between clinical and environmental strains (p <0.05). Error bars represent 95% confidence intervals for the true mean expression.

4.4.3 There is a correlation between RND efflux pump expression and MIC.

E-test was used to determine MIC of gentamicin (GM), erythromycin (EM), ceftazidime (TZ), co-trimoxazole (TS), doxycycline (DC), co-amoxiclav (XL), chloramphenicol (CL), and levofloxacin (LE) in 33 *B. pseudomallei* strains randomly

chosen from the 60 strains used for the expression analysis. The MIC of trimethoprim (TM) was only determined for nine strains. The strains used for antibiotic susceptibility testing and corresponding MICs for the antibiotics tested are recorded in **Table 4-4.** Linear regression and ANOVA were both used to compare expression of each pump to MIC for each antibiotic. Both the expression levels and the MICs for each strain occurred over a large range; therefore, for linear regression, both the MIC and expression levels were converted to a $\log_2$ scale to facilitate analysis. In ANOVA expression levels were also $\log_2$ transformed, for the same reason. Only those relationships with a p-value <0.05 were considered significant. p-values for linear regression and ANOVA are summarized in **Table 4-5.**

Expression levels of each efflux pump were plotted against the MIC of each antibiotic and relationships were analyzed by linear regression. Six relationships were recognized as significant using linear regression. As shown in **Figure 4-10,** *bpeH* and *BPSL0309* both have a positive correlation to gentamicin MIC; as expression of these two pumps increases, the MIC of gentamicin increases.

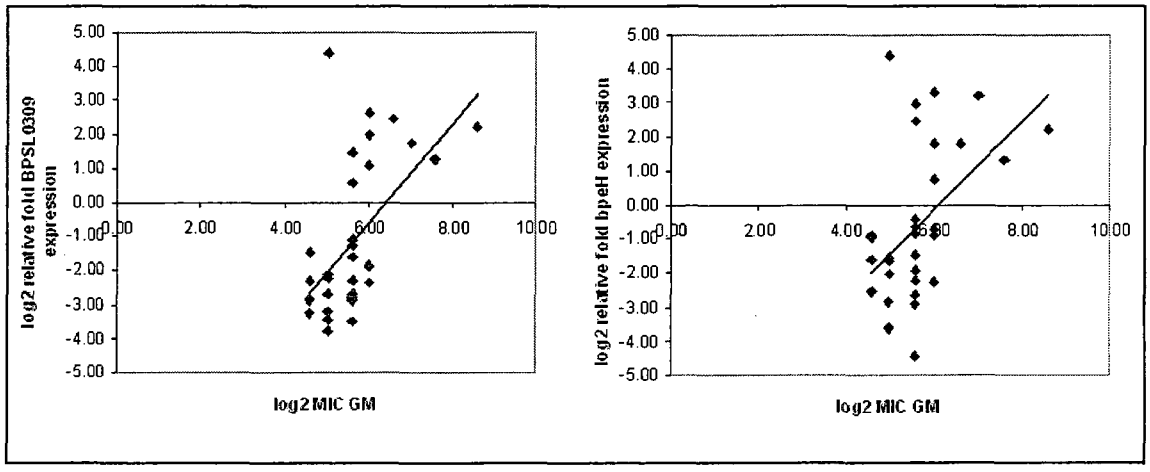

Figure 4-10 Linear regression plots of *BPSL0309* and *bpeH* expression compared to MIC of GM

Table 4-4 MIC for select Thai *B. pseudomallei* isolates. Gentamicin (GM), erythromycin (EM), ceftazidime (TZ), co-trimoxazole (TS), doxycycline (DC), co-amoxiclav (XL), chloramphenicol (CL), levofloxacin (LE), trimethoprim (TM), not determined (ND).

	MIC µg/mL								
Isolate Number	GM	EM	TZ	TS	DC	XL	CL	LE	TM
2	32	>256	1	0.125	0.75	1	4	1	ND
3	48	>256	1	0.25	1	1	4	1.5	ND
5	64	>256	1.5	0.38	0.5	1	4	1	ND
6	48	>256	2	0.25	1.5	1.5	8	1.5	ND
7	ND	ND	ND	ND	ND	ND	ND	ND	4
14	64	>256	1.5	0.5	0.75	1	6	1.5	>32
15	48	>256	1.5	0.19	0.5	1.5	6	1.5	ND
16	64	>256	1	0.75	0.75	2	8	2	ND
17	ND	ND	ND	ND	ND	ND	ND	ND	>32
19	ND	ND	ND	ND	ND	ND	ND	ND	4
23	64	>256	1	0.125	1	0.75	4	1.5	>32
26	48	>256	1	0.75	1	1.5	6	2	ND
27	24	>256	1.5	0.19	0.75	1	8	1	ND
28	128	>256	1	0.125	1	1.5	6	2	>32
29	48	>256	1.5	0.064	0.5	1	8	2	ND
30	48	>256	1.5	0.125	1	1	6	3	ND
31	64	>256	1	0.25	0.38	1	8	2	ND
32	96	>256	1	0.25	0.5	1	4	1.5	>32
34	48	>256	1	1.5	1	1.5	6	1.5	>32
35	48	>256	1	0.38	0.75	1.5	6	1	ND
39	24	>256	1	0.125	0.75	1.5	4	1	ND
40	32	>256	0.38	0.75	0.38	0.5	6	0.75	>32
41	ND	ND	ND	ND	ND	ND	ND	ND	>32
44	384	128	1.5	1.5	0.75	1.5	6	3	ND
46	24	48	1	0.5	0.75	1	4	0.75	ND
48	24	>256	1	0.19	0.5	1.5	4	2	ND
49	32	>256	1	0.75	0.75	1.5	6	1.5	ND
50	32	>256	1.5	0.5	0.5	1.5	3	2	ND
51	ND	ND	ND	ND	ND	ND	ND	ND	>32
52	48	>256	1	0.19	0.75	1	4	0.75	ND
53	192	>256	1.5	0.75	1.5	1.5	6	2	ND
54	48	>256	1.5	0.19	0.75	1	6	1.5	ND
55	24	>256	1.5	0.125	0.5	1.5	4	0.75	ND
56	48	>256	1	0.094	0.5	1	6	1.5	ND
57	32	>256	1	0.125	0.75	1.5	4	2	ND
58	32	>256	1.5	0.38	0.75	1.5	6	1.5	ND
59	64	>256	1	0.5	1.5	1.5	6	1.5	1.5
60	32	>256	1.5	0.19	0.75	1	4	1.5	ND

Inverse relationships were identified for *bpeB* and *BPSL1567* expression compared to MIC for co-trimoxazole; as expression of these two pumps increased, the MIC of co-trimoxazole decreased. Additionally, as expression of *BPSL0309* increased, the MICs of

Table 4-5 *p*-values from linear regression and ANOVA analysis of correlations between MIC and RND efflux pump expression. Significant *p*-values are boldfaced. Gentamicin (GM), erythromycin (EM), ceftazidime (TZ), co-trimoxazole (TS), doxycycline (DC), co-amoxiclav (XL), chloramphenicol (CL), levofloxacin (LE), trimethoprim (TM), not determined (ND).

	***p*-value**													
	amrB		***bpeB***		***bpeF***		***bpeH***		***BPSL0309***		***BPSL1267***		***BPSL1567***	
Antibiotic	Linear	ANOVA	Linear	ANOVA	Linear	ANOVA	Linear	ANOVA	Linear	ANOVA	Linear	ANOVA	Linear	ANOVA
GM	0.68	0.71	0.73	0.88	0.08	0.16	**0.00**	**0.03**	**0.00**	**0.01**	0.21	0.48	0.36	0.66
EM	0.53	0.65	0.82	0.84	0.91	0.80	0.70	0.41	0.77	0.33	0.96	0.97	0.83	0.95
TZ	0.18	0.45	0.39	0.36	0.07	0.34	0.10	0.62	0.05	0.48	0.19	0.34	0.34	0.35
TS	0.18	0.66	**0.03**	0.27	0.31	0.36	0.81	0.62	0.48	0.48	0.18	0.43	**0.03**	0.17
DC	0.94	0.19	0.59	0.19	0.95	**0.02**	0.75	**0.01**	0.86	**0.01**	0.92	0.23	0.99	0.14
XL	0.06	0.06	0.11	0.12	0.09	**0.02**	0.05	**0.01**	**0.03**	**0.01**	0.16	0.06	0.07	**0.03**
CL	0.77	0.49	0.31	0.23	0.88	0.94	0.20	0.44	0.35	0.63	0.49	0.56	0.57	0.62
LE	0.95	0.40	0.82	0.99	0.61	0.87	0.23	0.03	0.24	0.33	0.74	0.93	0.81	0.97
TM	0.56	0.45	0.83	0.85	0.61	0.66	0.77	0.85	0.47	0.49	0.96	0.95	0.87	0.91

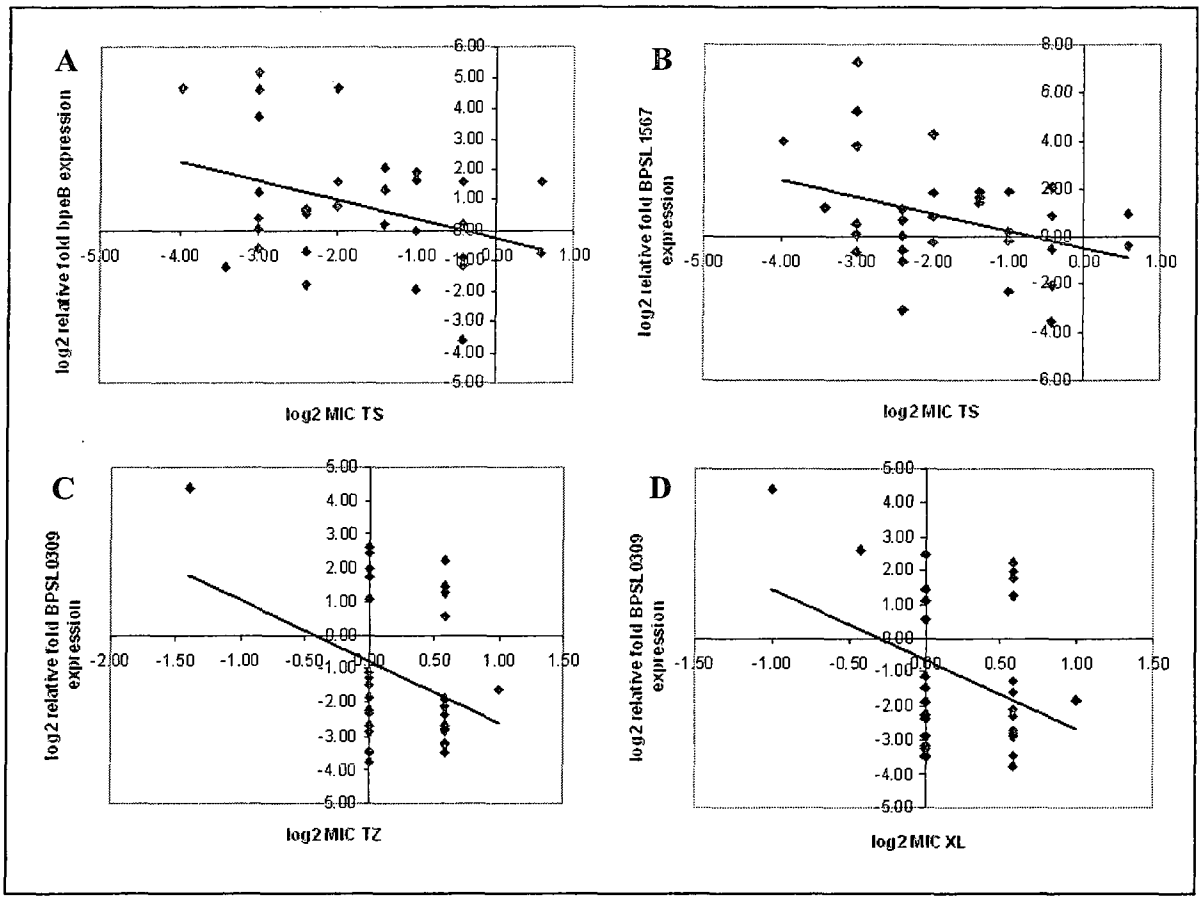

Figure 4-11 Linear regression plots for inverse relationships between RND efflux pump expression and MIC. Panel A represents the relationship between expression of *bpeB* and MIC of TS; Panel B represents the relationship between expression of *BPSL1567* and MIC of TS; Panel C represents the relationship between *BPSL0309* expression and MIC of TZ, and Panel D represents the relationship between *BPSL0309* expression and MIC of XL. Abbreviations: co-trimoxazole (TS), ceftazidime (TZ), co-amoxiclav (XL)

both co-amoxiclav and ceftazidime decreased. The linear regression plots illustrating these inverse relationships are shown in **Figure 4-11.** Using ANOVA, instead of being considered a continuous linear variable, MIC is considered a category. Thus expression levels are compared between categories of MIC. ANOVA indicated statistically significant associations between the expression of *bpeF, bpeH, BPSL0309,* and *BPSL1567*and the MIC of co-amoxiclav. Expression of both *BPSL0309* and *bpeH* had statistically significant correlations with the MIC of gentamicin, when analyzed by both linear regression and ANOVA. Least significant difference (LSD) and Waller-Duncan

are two statistical tests used to summarize the pairwise comparisons made in ANOVA. Both Waller-Duncan and LSD recognized that *bpeH* and *BPSL0309* expression levels in strains with MICs of <60 μg/mL for gentamicin were significantly lower than expression levels of strains with higher MICs. These relationships, along with all the other statistically significant correlations between MIC and efflux pump expression recognized by ANOVA are shown in **Figure 4-12** through **4-14.** These graphs show $\log_2$ mean relative fold RND efflux pump expression at each MIC along with the 95% confidence interval for expression at each MIC.

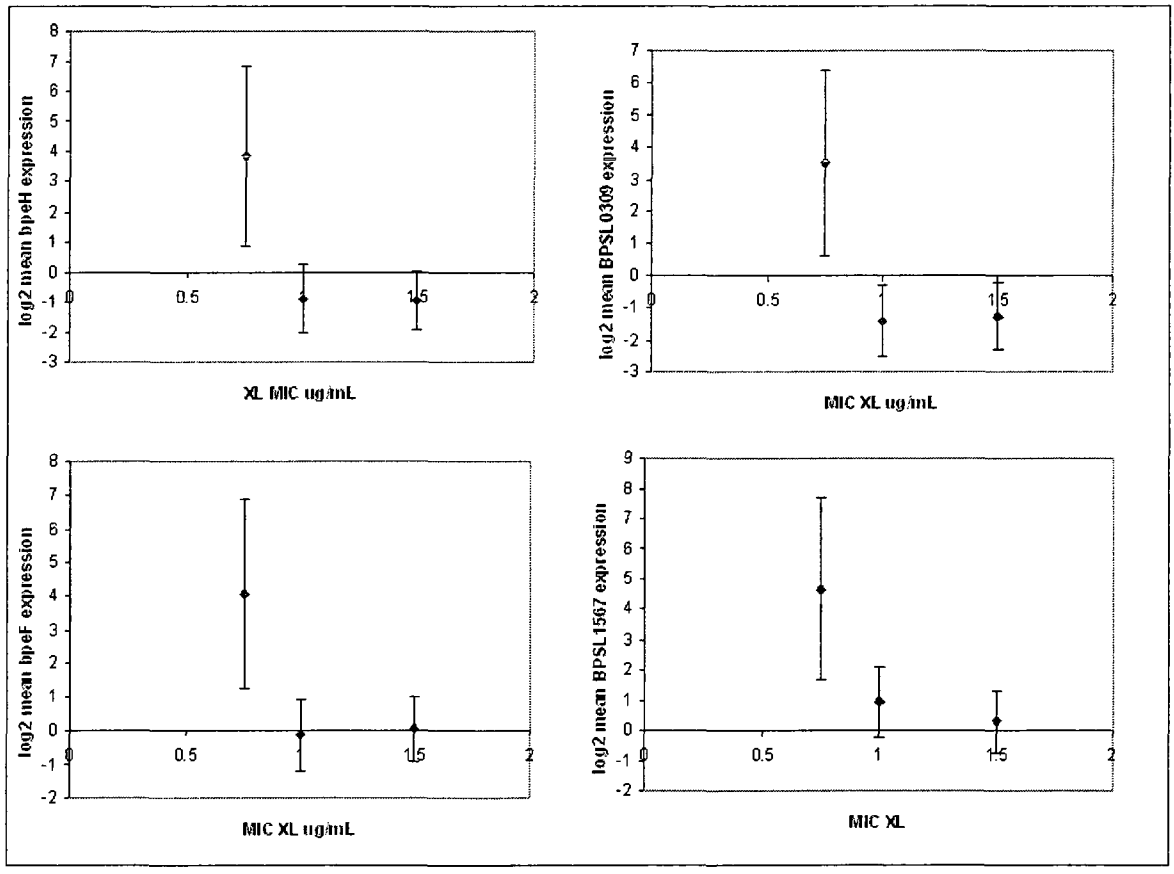

Figure 4-12 Expression levels of *bpeH, bpeF, BPSL0309,* and *BPSL1567* correlate to MIC of co-amoxiclav. For this analysis, all strains with MIC ≤0.75 μg/mL were grouped together and graphed at 0.75, likewise, all strains of MIC ≥1.5 μg/mL were grouped together at 1.5. Strains with MIC ≤0.75 μg/mL for co-amoxiclav had higher efflux pump expression than strains with MICs of 1 μg/mL or greater. Although these relationship were found to be statistically significant, *n* for MIC ≤0.75 μg/mL was only 2.

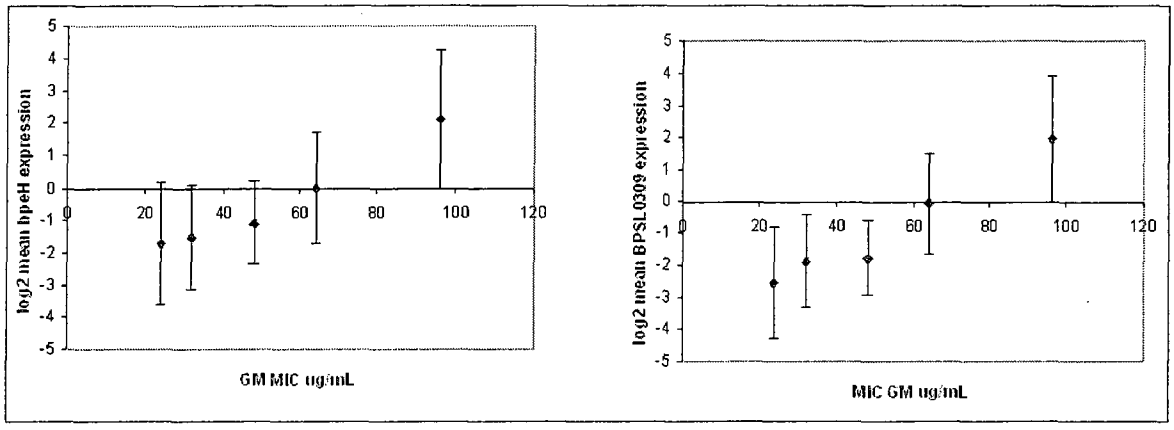

Figure 4-13 Expression levels of *BPSL0309* and *bpeH* correlate to MIC of gentamicin. For this analysis, strains with a MIC for gentamicin of 96 μg/mL or greater were considered as one group which is graphed at 96 μg/mL. Strains with MIC of gentamicin <60 μg/mL have significantly different expression levels of both *BPSL0309* and *bpeH* when compared to strains with MICs of 96 μg/mL or greater.

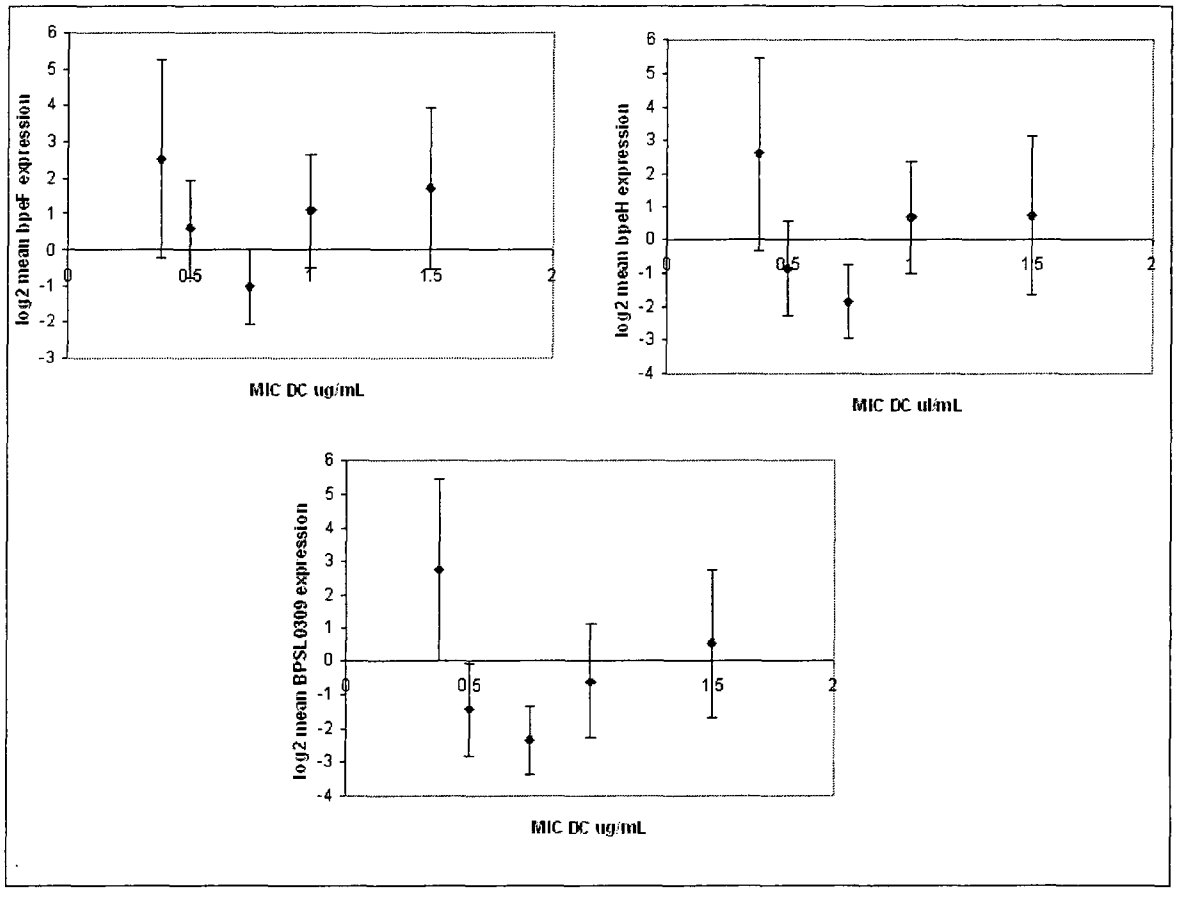

Figure 4-14 Expression of *bpeF, bpeH,* and *BPSL0309* correlate to MIC of doxycycline. Expression levels of these three pumps are significantly lower in srtrains with an MIC for doxycycline of 0.75 μg/mL than in strains with an MIC of 0.38 μg/mL or 1.5 μg/mL. High expression levels for strains with an MIC of 0.38 μg/mL may be misleading as n=2 for this group.

4.5 Discussion

At least 10 RND efflux pumps have been predicted in *B. pseudomallei*. All three of the pumps that have been characterized have displayed the ability to confer high level resistance to clinically significant drugs (aminoglycosides, macrolides, tetracyclines, chloramphenicol, and trimethoprim). However, this characterization has been accomplished in only a limited number of strains, and whether these strains are representative of wild type *B. pseudomallei* populations remains unclear. Here, we have analyzed the expression of seven RND efflux transporters (characterized: *amrB, bpeB, bpeF* predicted: *bpeH, BPSL0309, BPSL1267, BPSL1567)* in 60 strains of *B. pseudomallei* from Thailand using *q*RT-PCR. Only nine of the sixty strains tested had little or no expression of the seven efflux pumps analyzed, suggesting that expression of all pumps is widespread in both clinical and environmental *B. pseudomallei* isolates from Thailand.

Comparison of efflux pump expression in clinical and environmental strains revealed that *bpeH* was expressed at a significantly higher level in clinical strains. The majority of clinical isolates used for this study were primary isolates and therefore had not already been exposed to antibiotics. Thus exposure to antibiotics and subsequent selection of isolates over expressing this pump is an unlikely explanation for the observation that *bpeH* is more highly expressed in clinical isolates. However, over expression of RND efflux pumps *in vivo* in the absence of antibiotics has been observed in *Pseudomonas areuginosa* (17). Additionally, efflux deficient mutants of *P. aeuginosa*, *Salmonella enterica,* and *Vibrio cholerae* as well as a *B. pseudomallei bpeAB-oprB* mutant were all attenuated *in vivo* suggesting efflux pumps may play a role as virulence determinants (4, 5, 12). Although the exact contribution of these pumps to

virulence is unclear, current speculation includes extrusion of quorum sensing molecules necessary for coordination of colonization and protection from host defenses such as bile salts and long chain fatty acids (3, 6, 19, 23), Increased expression of *bpeH* in clinical isolates may indicate this pump's potential role as a virulence determinant. The other six efflux pumps analyzed were not differentially expressed in clinical and environmental isolates under the conditions tested in this study. However, because expression in all strains was tested under laboratory conditions, the possibility still exists that the pumps would be differentially expressed *in vivo* or under other conditions *B. pseudomallei* may encounter in its environmental niche.

Aminoglycoside resistance in *B. pseudomallei* has been attributed to efflux by both AmrAB-OprA and BpeAB-OprB (7, 28). Although aminoglycosides have been identified as substrates for pumps in other bacteria, such as the MexAB-OprM and MexXY-OprM efflux systems of *P. aeruginosa*, the AmrAB-OprA efflux pump of *B. pseudomallei* is unique in its ability to confer high level resistance to this group of antimicrobials (13, 25, 28). Expression of both *bpeH* and *BPSL0309* had a statistically significant correlation to increased resistance to gentamicin by both linear regression and ANOVA. This may indicate that aminoglycosides may also be substrates for these two pumps. Alternatively, expression of these pumps may be co-regulated with other resistance determinants that confer resistance to gentamicin, such as AmrAB-OprA. In *Bacillus subtilis*, Mta, a global regulator, activates expression of two different efflux pumps, Bmr and Blt (1). Likewise, the global regulator MarA controls expression of three efflux pumps in *Escherichia coli*: YadGH (ABC), YdeA (MFS), and AcrAB-TolC (RND) (2).

The inverse relationships between expression of *bpeB, BPSL1567,* and *BPSL0309* and resistance to co-trimoxazole (for the first two) and ceftazidime and co-amoxiclav (for *BPSL0309)* seems counterintuitive, as one would expect increased resistance in response to increased efflux expression. While this relationship indicates that these antimicrobials are not substrates for these three pumps, it may also indicate shared regulation between these three pumps and the resistance mechanisms for these three antimicrobials. For instance, it is possible that co-trimoxazole, ceftazidime, and co-amoxiclav are substrates for pumps besides *BPSL0309, BPSL1567*, and *bpeB* and these pumps are inversely regulated. Although the exact mechanism for regulation is unclear, inverse expression of efflux pumps has been observed in *P. aeruginosa* where both MexCD-OprJ and MexEF-OprN are inversely expressed with regard to MexAB-OprM (24). Identification of the mechanisms responsible for ceftazidime and co-trimoxazole resistance is imperative, as these are first line treatments for *B. pseudomallei* infections. The apparent relationship between co-amoxiclav susceptibility and expression of *bpeF, bpeH, BPSL0309,* and *BPSL1567* is probably false, as there were only two strains with an MIC $\leq$0.75 μg/mL and this may have skewed the statistical analysis. In order to confirm this relationship, a larger number of strains must be analyzed.

Correlations between the MIC of doxycycline and expression of *bpeH, bpeF,* and *BPSL0309* were interesting in that initially, high expression of these pumps was correlated with susceptibility while subsequently there appeared to be a linear relationship where increasing expression of these pumps correlated to increasing resistance to doxycycline. The initial correlation of higher expression to susceptibility may be misleading because, again, the small number of isolates with an MIC for

doxycycline ≤0.38 μg/mL may have skewed the statistical analysis. Aside from that small group of strains, there was a clear association between increased expression of *bpeH, bpeF,* and *BPSL0309* and increased resistance to doxycycline. This may indicate that doxycycline is a substrate for these three pumps. *bpeF* has been characterized in a surrogate strain, however, whether doxycycline was a substrate was not assessed (20).

Here we have demonstrated that RND efflux expression is widespread in clinical and environmental isolates from Thailand. Additionally, we have identified a putative efflux pump, *bpeH*, as being more highly expressed in clinical isolates. Correlations between gentamicin and doxycycline resistance and expression of *bpeH, bpeF,* and *BPSL0309* suggest that these antibiotics are potential substrates for these pumps. Furthermore, we identified correlations between expression of putative efflux pumps and susceptibility to co-trimoxazole and ceftazidime, which may help to elucidate mechanisms of resistance to these clinically important antimicrobials. Although disappointing, it was not surprising that we could not correlate expression of characterized efflux pumps and resistance to their known substrates. The literature indicates that these pumps have overlapping substrates, thus, resistance is a multifactoral characteristic and deficits in expression of one resistance determinant may be compensated by up-regulation of another. This work supports the notion that further characterization of RND efflux pumps will contribute to an overall understanding of the mechanisms of intrinsic resistance of *B. pseudomallei* which will facilitate development of improved treatment options for melioidosis.

4.6 References

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CHAPTER 5

DEVELOPMENT OF A *Burkholderia thailandensis* SURROGATE STRAIN USED FOR SCREENING INHIBITORS OF *Burkholderia pseudomallei* RND EFFLUX PUMPS

5.1 Abstract

Expression of at least two known efflux pumps, AmrAB-OprA and BpeAB-OprB significantly contribute to the intrinsic resistance of *Burkholderia pseudomallei*, thereby narrowing the spectrum of antimicrobials clinically relevant in treating infections with this organism. Inhibitors of these pumps, given in combination with antimicrobials otherwise ineffective against *B. pseudomallei*, would drastically increase the treatment options for these infections. Screening for efflux pump inhibitors in *B. pseudomallei* is problematic due to the strict guidelines set for working with this organism due to its status as a category B select agent. In order to facilitate this process, we have designed a panel of three surrogate *B. thailandensis* strains expressing AmrAB-OprA, BpeAB-OprB, or a control strain which expresses neither. These strains lack the *B. thailandensis* homologues of these pumps, thus observed phenotypes are directly related to expression of AmrAB-OprA and BpeAB-OprB from *B. pseudomallei*. When expressed in the

surrogate background, these pumps maintain their native substrate profiles. Additionally, pumps expressed in the surrogate background are inhibited by phenothiazines similar to the manner which has been previously described in *B. pseudomallei.* As *B. thailandensis* is not subject to select-agent regulations and can be manipulated under biosafety level 2 (BSL2) conditions, these strains serve as a practical substitute for *B. pseudomallei* itself in screening for and initial characterization of efflux pump inhibitors.

5.2 Introduction

Burkholderia pseudomallei, the etiologic agent of melioidosis, is intrinsically highly resistant to a wide spectrum of antibiotics including penicillins third generation cephalosporins, fluoroquinolones, aminoglycosides, and macrolides (29, 32, 33). Due largely to this resistance, availability of this organism, and a relatively high rate of morbidity and mortality this organism has been listed by the Centers for Disease Control and Prevention as a category B select agent, establishing a priority for the expansion of basic research in this organism (23). The current treatment regimen for melioidosis includes 10-14 days intravenous ceftazidime followed by 12-20 weeks of oral trimethoprim-sulfamethoxazole with or without doxycycline (25). Institution of ceftazimde as a first line antibiotic in the treatment of melioidosis dramatically decreased mortality; however, given that this antibiotic is not available in a oral formulation and must be delivered intravenously; the cost associated with treatment is considerable (30). Additionally, due to the intracellular nature of *B. pseudomallei* a long course of oral eradication therapy is required to prevent relapse of the infection; this long course of treatment is not ideal because it (i) increases the likelihood for resistance to develop, (ii) is associated with a low level of patient compliance and (iii) contributes to the expense of

treatment (4). The cost and burden on resources inflicted by treatment of melioidosis necessitates evaluation of alternative treatment regimens which will be more practical both in the event of intentional release and in treatment of melioidosis in resource-poor regions of endemicity.

Quinolones and macrolides have been identified as possible options to improve treatment of melioidosis because of their high intracellular permeation, oral formulation, bactericidal affect, and efficacy against biofilms (5, 32). However, a clinical trial testing the efficacy of these antimicrobials for the eradication phase of melioidosis treatment found that they were actually associated with a higher rate of treatment failure than the current recommended treatment (5). Macrolides have been identified as substrates for two efflux pumps in *B. pseudomallei*, AmrAB-OprA and BpeAB-OprB (3, 22). Additionally, our lab has identified fluoroquinolones as substrates for both of these pumps (T. Mima and H.P. Schweizer, unpublished results). Thus, efflux is the most likely explanation for resistance of *B. pseudomallei* to quinolones and macrolides and inhibition of efflux would render these antibiotics effective. Recently, Chan et al. (2) described the potentiation of aminoglycosides, macrolides, and fluoroquinolones by the efflux pump inhibiting phenothiazines. Unfortunately, phenothiazines were required at clinically unachievable concentrations in order to be effective. Additionally, the mechanism of action for inhibition of efflux was most likely disruption of the proton gradient; a target which does not confer selective toxicity and is therefore an undesirable characteristic of an efflux pump inhibitor.

Despite phenothiazines specifically being poor efflux pump inhibitors, Chan et al. have demonstrated that inhibition of AmrAB-OprA, BpeAB-OprB, and perhaps some

uncharacterized efflux pumps can be achieved, and more importantly, this inhibition leads to susceptibility of *B. pseudomallei* to drugs that would be clinically useful were it not for their extrusion by these pumps. Therefore, further development of efflux pump inhibitors effective against AmrAB-OprA and BpeAB-OprB has been suggested. Previously, such inhibitors have been identified via high through-put screening of small molecule libraries and further subsequent characterization and modification of promising lead compounds (11, 17, 18, 26). However, because of the restrictions placed on the conditions under which *B. pseudomallei* can be manipulated, high through-put screening with this organism is not feasible. Therefore, using *B. thailandensis,* a non-pathogenic non-select agent relative of *B. pseudomallei*, we have constructed a surrogate strain with inducible expression of *amrAB-oprA* and *bpeAB-orpB*. Because *B. thailandensis* can be manipulated under biosafety level 2 conditions, the strains described here are a good tool for high through-put screening of small molecule libraries to identify efflux pump inhibitors that can be used to potentiate otherwise useless antimicrobials in the treatment of melioidosis.

5.3 Materials and methods

5.3.1 Bacterial strains and media

B. thailandensis and *Escherichia coli* strains used in this study are listed in **Table 5-1**. All bacteria were routinely grown with aeration at 37°C. Low salt (5 g/L NaCl) Lennox LB broth (LSLB) and agar (MO BIO Laboratories, Carlsbad, CA) were used as rich media. Unless otherwise noted, antibiotics were added at the following concentrations: 100 μg/ml ampicillin (Ap), 15 μg/ml gentamicin (Gm), 35 μg/ml kanamycin (Km) and 25 μg/ml zeocin (Zeo) for *E. coli*; 500 μg/ml Km and 2,000 μg/ml Zeo for wild-type *B.*

thailandensis as well as Bt42. 200 μg/ml Zeo was used for Bt36 and Bt34. Tc 0.5 μg/ml and Km 5 μg/ml were used for Bt45. Gm 10 μg/ml and Km 5 μg/ml were used for Bt 43

Table 5-1 Strains, plasmids, and primers used in this study. Abbreviations: r, resistance; Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Zeo, zeocin. [b] P_{tac}, *E. coli lac/trp* operon hybrid promoter. Oligonucleotides were purchased from IDT, Coralville, IA.

Strain or plasmid	Relevant properties[a]	Reference or source
B. thailandensis		
E264	environmental isolate	[1]
Bt36	E264 with Δ(*amrAB-oprA*)::*FRT*	This study
Bt34	E264 with Δ(*bpeAB-oprB*)::*FRT*	This study
Bt38	Bt34 with Δ(*amrAB-oprA*)::*FRT*	This study
Bt43	Bt38::mini-Tn*7*T-LAC	This study
Bt44	Bt38:: mini-Tn*7*T-LAC-*amrA*$^+$*B*$^+$-*oprA*$^+$	This study
Bt45	Bt38:: mini-Tn*7*T-LAC-*bpeA*$^+$*B*$^+$-*oprB*$^+$	This study
E. coli		
DH5α	F$^-$ φ80Δ*lacZ*M15 (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	(16)
SM10(λ*pir*$^+$)	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r (λ*pir*$^+$)	(21)
RHO3	a Km susceptible, Δ*asd* derivative of SM10(λ*pir*$^+$)	Rholl and Schweizer, unpublished
Plasmids		
pGEM-T Easy	Ap^r; TA cloning vector	Promega
pPS2505	Ap^r; pGEM-T Easy with Δ(*amrAB-oprA*) PCR fragment	This study
pPS2506	Ap^r; pGEM-T Easy with Δ(*bpeAB-oprB*) PCR fragment	This study
pPS2509	Ap^r, Km^r; pPS2505 with *Hind*III *FRT-nptII-FRT* fragment from pFKM2	This study
pPS2508	Ap^r, Km^r; pPS2506 with *Kpn*I *FRT-nptII-FRT* fragment from pFKM2	This study
pEXKm4	Km^r; gene replacement vector with *gusA*	Lopez and Schweizer, unpublished
pPS2515	Km^r; pExKm4 with 2.2 Kb *Eco*RI Δ(*amrAB-oprA*)::*FRT-nptII-FRT* fragment from pPS2509	This study

pPS2514	Kmr; pExKm4 with 2.1 Kb EcoRI Δ(*bpeAB-oprB*)::*FRT-nptII-FRT* fragment from pPS2508	This study
pUC18T-mini-Tn7T-LAC	Ap^r, Gm^r; mini-Tn7 cloning and delivery vector	[2]
pPS2142	Ap^r, Gm^r; pUC18T-miniTn7T-LAC with $amrA^+B^+$-$oprA^+$; $amrA^+B^+$-$oprA^+$expression under P_{tac}[b] control	[3]
pPS2014	Ap^r, TC^r; pUC18T-miniTn7T-LAC with $bpeA^+B^+$-$oprB^+$; $bpeA^+B^+$-$oprB^+$ expression under P_{tac}[b] control	H. Blair, unpublished
pBADSce	Zeo^r; source of I-SceI	Lopez and Schweizer, unpublished
pTNS3	Ap^r; source of Tn7 TnsABCD transposition proteins	[3]
pFKM2	Ap^r Km^r; source of *FRT-nptII-FRT* cassette	[3]
pRK2013	Km^r; conjugation helper strain	(10)
pFLPe2[b]	Zeo^r; source of Flpe recombinase	[3]
pFLPe4[b]	Km^r; source of Flpe recombinase	[3]
Primers		
1889	5'- catgcgcgtcgaacgggttc	This study
1890	5'- cgcgagattcgcctgcgcc	This study
1891	5'- cgccttgccgtagtcgac	This study
1892	5'- ggcgcaggcgaatctcgcggtaccacgagaaggcgatcc	This study
1893	5'- ggtcgagcgacttgcgag	This study
1894	5'- gatcgatcctgaacagcacc	This study
1895	5'- cgtgatggcgttcgtgagc	This study
1896	5'- ggtgctgttcaggatcgatcctcaagcttgtggacgttcg	This study
618	5'- gttcgtcgtccactgggatca	(7)
619	5'- agatcggatggaattcgtggag	(7)
479	5'- attagcttacgacgctacaccc	(7)

and Gm10 μg/ml and Km100 μg/ml were used for Bt 44. Polymyxin B (PB) 15 μg/ml was used for counter-selection of *E. coli* when RHO3 was not the mobilizer strain. Growth of *E. coli* RHO3 required supplementation with 400 μg/ml diaminopimelic acid

(DAP; Sigma). Antibiotics were either purchased from Sigma, St. Louis, MO (ampicillin, kanamycin, polymyxin B and tetracycline), EMD Biosciences, San Diego, CA (gentamicin), or Invitrogen, Carlsbad, CA (zeocin).

5.3.2 DNA manipulations

Published procedures were employed for manipulation of DNA, and transformation of *E. coli* and *B. thailandensis* (6, 27, 28). Plasmid DNAs were isolated from *E. coli* using the QIAprep Mini-spin kit (Qiagen, Valencia, CA). Colony PCR with *B. pseudomallei* was performed as previously described (6). Custom oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

5.3.3 Construction of a recombinant plasmid for deletion of *amrAB-oprA* (*BTH_I2443-BTH_I2445* and *bpeAB-oprB* (*BTH_I0680-BTH_I0682*)

For construction of a Δ(*amrAB-oprA*) vector, ~317 bp region of *oprA* was amplified with primers 1893 and 1894 and 364 bp region of *amrA* was amplified with primers 1895 and 1896 using Platinum *Taq* HiFi DNA polymerase (Invitrogen, Carlsbad, CA) and *B. thailandensis* genomic DNA as template. These fragments were purified from an agarose gel using the Fermentas DNA Extraction Kit (Glen Burnie, MD) and used as template in a second round of overlap extension PCR using primers 1893 and 1895. The resulting 681 bp fragment was ligated into pGEM-T Easy (Promega) to yield pPS2505. The 1,514 bp *Hin*dIII fragment from pFKM2 containing the *FRT-nptII-FRT* cassette was ligated into *Hin*dIII digested pPS2505 (*Hin*dIII site was introduced by primer 1896 during PCR) yielding pPS2509. The 2.2 kb Δ(*amrAB-oprA*)::*FRT-nptII-FRT* mutation cassette was cut from pPS2509 with *Eco*RI and ligated into pEXKm4 to yield pPS2515.

For construction of Δ(*bpeAB-oprB*) vector, 352 bp fragment of *bpeA* was amplified using primers 1889 and 1890 and a 343 bp region of *oprB* was amplified with

primers 1891 and 1892 using Platinum *Taq* HiFi DNA polymerase and *B. thailandensis* E264 genomic DNA as template. These fragments were purified from an agarose gel using the Fermentas DNA extraction kit and used as template in a second round of overlap extension PCR using primers 1889 and 1891. The resulting 695 bp PCR product was cloned into pGEM-T Easy yielding pPS2506. The 1.4 kb *Kpn*I fragment from pFKM2 containing the *FRT-nptII-FRT* selection cassette was ligated into *Kpn*I digested pPS2506 (*Kpn*I site was introduced by primer 1892 during PCR) yielding pPS2508. The 2.1 kb Δ(*bpeAB-oprB*)::*FRT-nptII-FRT* deletion cassette was cut from pPS2508 with *Eco*RI and ligated into pEXKm4 to yield pPS2514.

5.3.4 I-*Sce*I mediated deletion of efflux operons

I-*Sce*I mediated allelic replacement procedure for *Burkholderia* sp. was developed by Lopez and Schweizer (unpublished). Briefly, the suicidal allelic exchange vector (pPS2514 and pPS2515) was introduced into *B. thailandensis* E264 via conjugation. *E. coli* RHO3 counter-selection was achieved by omitting the addition of DAP to the media. *B. thailandensis* merodiploid cells are selected using either the kanamycin resistance marker on the backbone of pEXKm4, or a resistance marker introduced in the deletion cassette (pPS2514 and pPS2515 both contain Km^r selection markers in the deletion cassette). Additionally, *gusA* on the backbone of pEXKm4 serves as a colorimetric indicator of merodiploid cells in the presence of its substrate, 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (x-Gluc; Gold Biotechnology St. Louis, MO). Blue merodiploids were electroporated with the I-*Sce*I expression vector, pBADSce, and zeocin resistant mutants were selected in the presence of 0.2% arabinose and 50 μg/ml x-Gluc. Marked mutants were identified as Km^r white colonies and mutations were

confirmed by PCR with either primers 1889 and 1891 or 1893 and 1895. The resistance cassette was then excised from the chromosome using pFlpe2 for Flp mediated recombination as described previously (7). The result was strains Bt34, Bt36, Bt38 containing unmarked deletions of *amrAB-oprA* (Bt36), *bpeAB-oprB* (Bt34) and both *amrAB-oprA* and *bpeAB-oprB* (Bt38).

5.3.5 Site-specific transposition of mini-Tn*7* elements and Flp excision of chromosomally integrated antibiotic resistance markers

Isolation of chromosomally-integrated mini-Tn*7* elements followed by Flp-mediated selection marker excision was performed using recently published procedures (6). Briefly, pUC18T-mini-Tn*7*T-LAC, pPS2142 or pPS2014 were introduced into Bt38 via conjugation. pUC18T-mini-Tn*7*T-LAC was mobilized into *B. thailandensis* using *E. coli* SM10(λpir^+), for which counter-selection was achieved with 15 μg/ml PB. pPS2014 harbored in *E. coli* DH5α was mobilized using helper plasmid pRK2013 into *B. thailandensis* Bt38; again 15 μg/ml PB was used for counter-selection of *E. coli*. pPS2142 in *E. coli* RHO3 was conjugated with *B. thailandensis* Bt38; counter-selection of *E. coli* was achieved by omission of DAP from the media. All conjugations also included pTNS3 (in *E. coli* RHO3) which was the source of the enzymes required for site specific transposition. Transformants were selected with either Gm or Tc and screened by colony PCR for insertions downstream of either *glmS*1 or *glmS*2 using primers 618 and 479 or 619 and 479, respectively as described in (7). Isolates with insertions at *glmS*1 were chosen for further characterization. The resistance cassette was then excised from the chromosome using pFlpe4 for Flp mediated recombination as described previously (7). This resulted in *B. thailandensis* Bt38 strains with unmarked insertions of (i) an empty mini-Tn*7* cassette (Bt43) (ii) mini-Tn*7*-$amrA^+B^+$-$oprA^+$ from *B.*

pseudomallei 1026b driven by the inducible promoter, P_{tac} (Bt44) or (iii) mini-Tn7-*bpeA*$^{+}$*B*$^{+}$-*oprB*$^{+}$ from *B. pseudomallei* 1026b driven by the inducible promoter, P_{tac} (Bt45).

5.3.6 Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) were determined in Mueller-Hinton broth from Becton Dickinson (Franklin Lakes, NJ) by the two-fold broth microdilution technique following Clinical and Laboratory Standards Institute guidelines (9). All MICs for strains with mini-Tn*7* insertions were performed in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The MICs were recorded after incubation at 37°C for 15 to 16 h.

5.3.7 Checkerboard titration assays

Checkerboard assays to determine synergistic effects of varying combinations of efflux pump inhibitor and antibiotic concentrations were performed as described previously (17). Potentiation of Gm, clarithromycin (Clr), clindamycin (Cld), ceftazidime (Cef) and norfloxacin (Nor) by either promazine (PMZ; Sigma) or chlorpromazine (CPZ, Sigma) was measured in 96-well format. MICs of each antibiotic were measured in the presence of PMZ or CPZ at concentrations ranging from 1000 μM in 2 fold series dilutions to 15.6 μM.

5.4 Results

5.4.1 *B. thailandensis* E264 has *amrAB-oprA* and *bpeAB-oprB* homologues

In silico analysis of the *B. thailandensis* E264 genome identified *BTH_I2445-BTH_I2444-BTH_I2443* and *BTH_I0680-BTH_I0681-BTH_I0682* as homologues of *B. pseudomallei's amrAB-oprA* and *bpeAB-oprB*, respectively. *BTH_I2445-BTH_I2444-*

BTH_I2443 shares 95% overall nucleotide identity with the *B. pseudomallei* K96243 *amrAB-oprA* sequence while *BTH_I0680-BTH_I0681-BTH_I0682* shares 94% overall nucleotide identity with *bpeAB-oprB* from the same strain. *BTH_I2445-BTH_I2444-BTH_I2443* had previously been characterized as an *amrAB-oprA* homologue (1). Until this point, the *B. thailandensis* homologue of *bpeAB-oprB, BTH_I0680-BTH_I0681-BTH_I0682*, has not been characterized.

5.4.2 *B. thailandensis* Δ*amrAB-oprA*, Δ*bpeAB-oprB* and Δ*amrAB-oprA* Δ*bpeAB-oprB* mutants are susceptible to many antibiotics

In order to construct a *B. thailandensis* strain in which *amrAB-oprA* and *bpeAB-oprB* from *B. pseudomallei* could be expressed and analyzed, we deleted the *amrAB-oprA* and *bpeAB-oprB* homologues from *B. thailandensis* using a I-*Sce*I homing endonuclease based gene replacement system, as illustrated in **Figure 5-1** for deletion of *amrAB-oprA*. Deletion of *amrAB-oprA* resulted in susceptibility of the resulting strain, Bt36, to aminoglycosides and macrolides (**Table 5-2)** as previously reported for disruption of *amrAB-oprA* in *B. pseudomallei* 1026b (22). Additionally, we identified doxycycline and acriflavin as AmrAB-OprA substrates (**Table 5-2).** The MICs of norfloxacin, chloramphenicol, tetracycline, carbenicillin and ceftazidime were not affected by the deletion of *amrAB-oprA* in *B. thailandensis* E264.

Deletion of *bpeAB-oprB* resulted in increased susceptibility of the resulting strain, Bt34, to norflaxacin, tetracycline, doxycycline, and acriflavin; indicating that these antimicrobials are substrates for this pump. Furthermore, the *B. thailandensis* E264 Δ*amrAB-oprA* Δ*bpeAB-oprB* double mutant, Bt38, showed increased susceptibilities to macrolides, indicating that these antibiotics are also BpeAB-OprB substrates (**Table 5-2**). Aminoglycosides, carbenicillin, chloramphenicol and ceftazidime were not substrates for

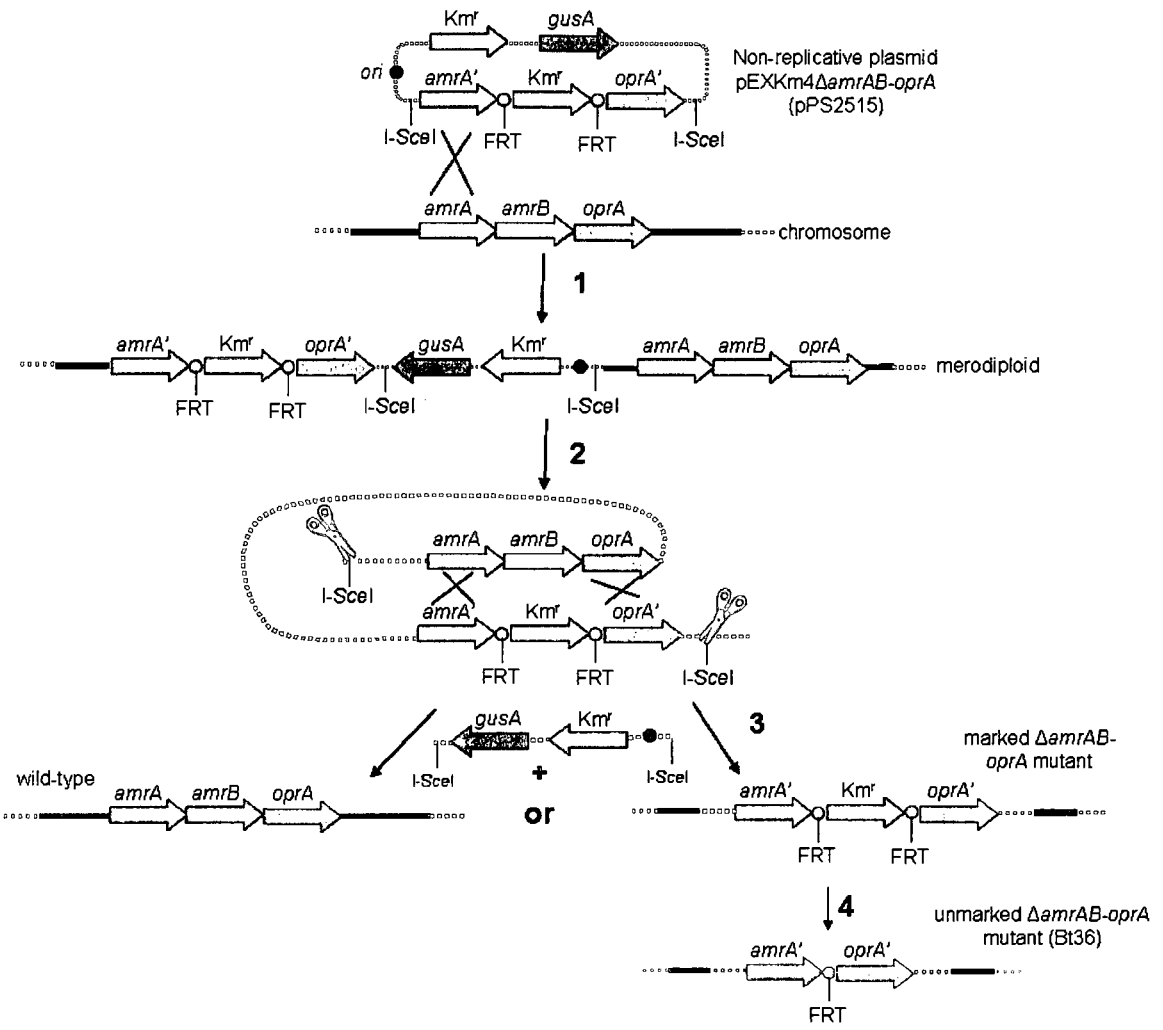

Figure 5-1 Schematic depicting deletion of *amrAB-oprA* using I-SceI mediated gene replacement system. **(1)** Non-replicative allelic replacement plasmid, pPS2515, was introduced into *B. thailandensis* E264 via conjugation. Merodiploid transformants were selected using kanamycin and the colorimetric indicator x-Gluc (substrate of *gusA*); merodiploids were blue Km^r colonies. **(2)** pBADSce, the source of the homing endonuclease I-*Sce*I was electroporated into a merodiploid cell. The endonuclease introduces site-specific double stranded breaks in the DNA which induces homologous recombination. Transformants were selected using zeocin (the resistance marker on pBADSce), x-Gluc, and 0.2% arabinose (to induce expression of I-*Sce*I). Transformants were white Zeo^r colonies. **(3)** Following counter-selection with I-*Sce*I, the pPS2515 plasmid backbone is lost from the chromosome, leaving a mixed population of colonies that have either reverted to wild-type or are mutants (*ΔamrAB-oprA::FRT-Km-FRT*). To screen for mutants, individual colonies were patched on media containing Km and x-Gluc. Mutants were white Km^r colonies. **(4)** Using Flp-mediated recombination, the Km^r marker was excised from the chromosome, resulting in an unmarked *ΔamrAB-oprA* mutant, Bt36. Abbreviations: Km, kanamycin; *amrA'*, ~300 bp region of homology to *amrB*; *oprA'*, ~300 bp region of homology to *oprA*.

BpeAB-OprB. Our results are in contrast to previous reports identifying aminoglycosides and macrolides but not quinolones or tetracyclines as substrates for BpeAB-OprB in *B. pseudomallei* KHW (3).

Table 5-2 Susceptibilities of *B. thailandensis* strains to antimicrobial agents. E264 is wild type *B. thailandensis* and the parental strain of Bt36, Bt 34, and Bt38. Bt36 is *B. thailandensis* E264 Δ(*amrAB-oprA*); Bt34 is *B. thailandensis* E264 Δ(*bpeAB-oprB*); Bt38 is *B. thailandensis* E264 Δ(*amrAB-oprA*) Δ(*bpeAB-oprB*) double mutant. A change in MIC ≥4 fold over the parental strain is considered significant, numbers meeting this criteria are displayed in boldface.

Antibiotic	MIC (μg/ml)			
	E264	Bt36	Bt34	Bt38
Gentamicin	1024	**1**	1024	**1**
Kanamycin	256	**1**	256	**1**
Erythromycin	>128	**8**	128	**1**
Clarithromycin	>128	**4**	64	**0.5**
Clindamycin	>128	>128	>128	**64**
Norfloxacin	16	8	**2**	**0.5**
Chloramphenicol	8	4	4	4
Tetracycline	2	1	**0.5**	**0.03**
Doxycycline	1	**0.25**	**0.25**	**0.008**
Carbenicillin	512	512	512	512
Ceftazidime	4	4	4	4
Acriflavin	64	**8**	**16**	**0.5**

5.4.3 Substrate profiles of AmrAB-OprA and BpeAB-OprB do not change when expressed in surrogate *B. thailandensis* strain.

Using the mini-Tn7 site specific integration system previously developed in our lab, the *amrAB-oprA* and *bpeAB-oprB* (both cloned from *B. pseudomallei* 1026b) operons were inserted into the *B. thailandensis* Bt38 chromosome at the *glmS*1-associated Tn*7* insertion site on chromosome 1 (7) (as illustrated in **Figure 5-2** for $amrA^{+}B^{+}$-$oprA^{+}$). Expression of *amrAB-oprA and bpeAB-oprB* is under the control of the inducible promoter, P_{tac}; therefore, all MICs were performed in the presence of 1 mM IPTG to induce expression of the pumps. Bt43 is a control strain which has a mini-Tn*7* vector insertion in the same position as Bt44 and Bt45, but with no resistance determinant (i.e. no efflux pump).

When *amrAB-oprA* from *B. pseudomallei* 1026b was expressed in the efflux pump deficient strain *B. thailandensis* Bt38, the MICs of gentamicin, kanamycin,

erythromycin, clindamycin, norfloxacin, tetracycline, doxycycline, and acriflavin increased anywhere from 16-1024 fold as compared to the control strain, *B. thailandensis* Bt43 (**Table 5-3**). The MICs of chloramphenicol, carbenicillin and ceftazidime are not

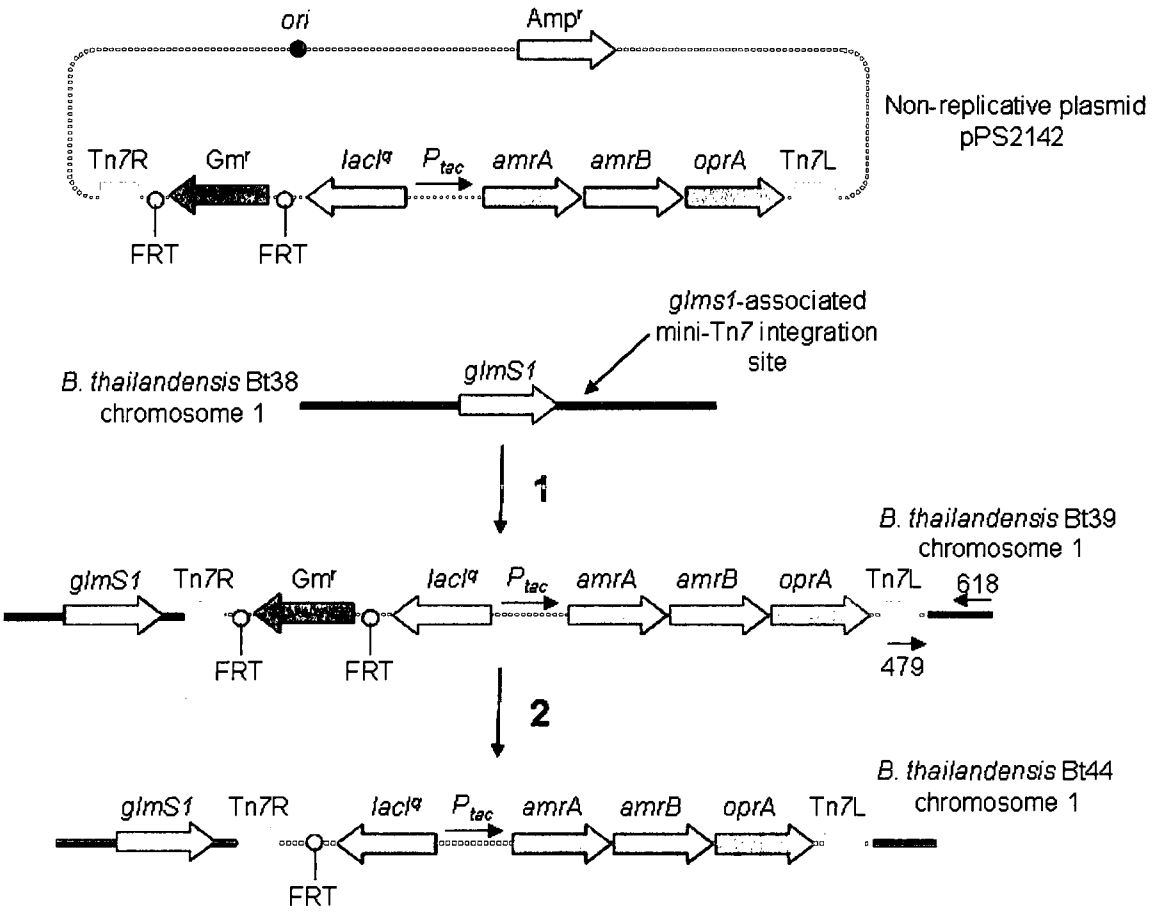

Figure 5-2 Schematic depicting site specific integration of *B. pseudomallei amrAB-oprA* into surrogate host strain, *B. thailandensis* Bt38. (1) The non-replicative mini-Tn*7*- *amrA*$^+$*B*$^+$-*oprA*$^+$ expression vector and pTNS3 (the source of Tn*7* TnsABCD transposition proteins) were simultaneously introduced into *B. thailandensis* BT38, an efflux deficient strain, via conjugation. Transformants were selected using gentamicin (Gm). There are two *glmS* genes in *B. thailandensis* and mini-Tn*7* integration can occur at the integration sites associated with either *glmS1* or *glmS2*. Screening for insertion at the *glmS1*-associated integration site was achieved by PCR with primers 618 and 479. **(2)** Using Flp-mediated recombination, the Gm^r marker was excised from the chromosome, the resulting strain, Bt44, contains an unmarked, single copy, site specific, insertion of *amrAB-oprA* into *B. thailandensis* Bt38. Expression of *amrAB-oprA* from P_{tac} can be induced with 1 mM IPTG.

affected by the expression of *amrAB-oprA*. MICs of the efflux double mutant expressing *amrAB-oprA* return to levels similar to that of wild type *B. thailandensis* E264 (**Table 5-3**).

When *bpeAB-oprB* from *B. pseudomallei* 1026b was expressed in the efflux pump deficient strain *B. thailandensis,* Bt38, the MICs of erythromycin, clarithromycin, clindamycin, norfloxacin, tetracycline, and doxycycline increased 4-32 fold compared to control strain *B. thailandensis* Bt43 (**Table 5-3**). MICs of gentamicin, kanamycin, chloramphenicol, carbenicillin, ceftazidime, and acriflavin did not increase when *bpeAB-oprB* was expressed in the *B. thailandensis* pump deficient strain, Bt38. Expression of *bpeAB-oprB* itself was not sufficient to return MICs of the pump deficient strain to wild type levels (**Table 5-3**).

Table 5-3 Susceptibilities of efflux pump deficient *B. thailandensis* strains expressing eitber *B. pseudomallei amrAB-oprA* or *bpeAB-oprB* to antimicrobial agents. E264 is wild type *B. thailandensis*. Bt43, Bt38::mini-Tn*7*T-LAC, is a pump deficient *B. thailandensis* control strain containing the empty expression vector. Bt 44, Bt38::mini-Tn*7*T-*amrA*$^+$*B*$^+$-*oprA*$^+$, is a pump deficient *B. thailandensis* strain expressing *amrAB-oprA* from *B. pseudomallei* 1026b. Bt45, Bt38::mini-Tn*7*T-bpe*A*$^+$*B*$^+$-*oprB*$^+$, is a pump deficient *B. thailandensis* strain expressing *bpeAB-oprB*. MICs of all strains were measured in the presence of 1 mM IPTG to induce expression of *amrAB-oprA* and *bpeAB-oprB* from P_{tac}. A change in MIC ≥4 fold over the control strain is considered significant, numbers meeting this criteria are displayed in boldface.

Antibiotic	MIC (μg/ml)			
	E264	Bt43	Bt44	Bt45
Gentamicin	1024	1	**1024**	1
Kanamycin	256	4	**256**	4
Erythromycin	>128	1	**>128**	**4**
Clarithromycin	>128	1	**>128**	**4**
Clindamycin	>128	64	**1024**	**512**
Norfloxacin	16	1	**16**	**8**
Chloramphenicol	8	2	4	2
Tetracycline	2	0.03	**2**	**0.25**
Doxycycline	1	0.008	**0.5**	**0.25**
Carbenicillin	512	512	512	512
Ceftazidime	4	4	4	4
Acriflavin	64	1	**32**	1

5.4.4 EPIs potentiate antibiotics in surrogate strains expressing *amrAB-oprA* and *bpeAB-oprB*

The potentiation of several antibiotics by the phenothiazines, promazine (PMZ) and chlorpromazine (CPZ), against *B. pseudomallei* KHW was recently described (2). We were interested in determining (i) whether phenothiazines were inhibiting either AmrAB-OprA or BpeAB-OprB and (ii) whether the same potentiating effects could be observed using a surrogate *B. thailandensis* background. To this end, we used checkerboard assays to measure the MICs of gentamicin, clarithromycin, clindamycin, norfloxacin and ceftazidime of strains Bt43, Bt44, and Bt45 in the presence of varying concentrations of the efflux pump inhibitors (EPIs), PMZ or CPZ.

Potentiation was measured as the fold difference between MIC of the antibiotic alone and MIC of the antibiotic in the presence of inhibitor (at concentrations where the EPI alone was not inhibitory). In the control strain, Bt43, no antibiotic was potentiated greater than 2 fold (**Table 5-4**); also, PMZ and CPZ alone were inhibitory at concentrations above 250 and 31.3 μM, respectively. When *amrAB-oprA* was expressed, as in Bt44, the MICs of gentamicin, clarithromycin, clindamycin, and norfloxacin were all decreased significantly (8 to >512 fold) in the presence of 250 μM PMZ (**Table 5-5**). CPZ decreased the MIC of gentamicin >16 fold in strain Bt44, but did not potentiate clarithromycin, clindamycin, norfloxacin, or ceftazidime beyond what was observed in control strain Bt43 (**Table 5-5**). For strain Bt44, PMZ and CPZ were inhibitory alone at concentrations greater than 500 and 250 μM, respectively. When *bpeAB-oprB* was expressed, as in Bt45, the MIC of clindamycin was decreased 4 fold in the presence of 250 μM PMZ and the MIC of norfloxacin was decrease 64 fold in the presence of 62.5 μM CPZ (**Table 5-6**). Other antibiotics were not potentiated by either PMZ or CPZ

Table 5-4 MICs of efflux deficient control strain, Bt43 in the presence of varying concentrations of the EPIs, PMZ and CPZ. The fold potentiation is the fold difference between MIC of the antibiotic in the absence of EPI and the MIC of the antibiotic in combination with the maximum concentration of EPI which is not inhibitory itself. MICs of the antibiotic when in combination with an EPI at a concentration above the MIC of the EPI alone are not synergistic, but are a reflection of complete inhibition by the EPI itself. Combinations of EPI and antibiotic where synergy is recognized are indicated in boldface.

Antibiotic	EPI	EPI MIC (μM)	MIC (μg/ml) of antibiotic at indicated concentration of EPI								Fold potentiation
			0	1000	500	250	125	62.5	31.3	15.6	
Gentamicin	PMZ	250	2	<0.03	<0.03	<0.03	**1**	2	2	2	2
	CPZ	31.3	2	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	2	0
Clarithromycin	PMZ	250	0.5	<0.25	<0.25	<0.25	0.5	0.5	0.5	0.5	0
	CPZ	31.3	0.5	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	0.5	0
Clindamycin	PMZ	250	32	<1	<1	<1	32	32	32	32	0
	CPZ	31.3	32	<1	<1	<1	<1	<1	<1	**16**	2
Norfloxacin	PMZ	250	0.25	<0.02	<0.02	<0.02	**0.125**	0.25	0.25	0.25	2
	CPZ	31.3	0.25	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	**0.125**	2
Ceftazidime	PMZ	250	4	<0.06	<0.06	<0.06	**2**	4	4	4	2
	CPZ	31.3	4	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	**2**	2

Table 5-5 MICs of *amrAB-oprA* expressing strain, Bt44 in the presence of varying concentrations of the EPIs, PMZ and CPZ. The fold potentiation is the fold difference between MIC of the antibiotic in the absence of EPI and the MIC of the antibiotic in combination with the maximum concentration of EPI which is not inhibitory itself. MICs of the antibiotic when in combination with an EPI at a concentration above the MIC of the EPI alone are not synergistic, but are a reflection of complete inhibition by the EPI itself. Combinations of EPI and antibiotic where synergy is recognized are indicated in boldface.

Antibiotic	EPI	EPI MIC	MIC of antibiotic at indicated concentration of EPI								Fold potentiation
			0	1000	500	250	125	62.5	31.3	15.6	
Gentamicin	PMZ	500	>1024	<1	<1	**2**	**64**	**128**	**256**	**256**	>512
	CPZ	250	>1024	<1	<1	<1	**64**	**256**	**256**	**256**	>16
Clarithromycin	PMZ	500	128	<0.5	<0.5	**16**	**64**	128	128	128	8
	CPZ	250	128	<0.5	<0.5	<0.5	**64**	**64**	128	128	2
Clindamycin	PMZ	500	1024	<1	<1	**32**	1024	1024	1024	1024	32
	CPZ	250	1024	<1	<1	<1	1024	1024	1024	1024	0
Norfloxacin	PMZ	500	8	<0.02	<0.02	**0.25**	8	8	8	8	32
	CPZ	250	8	<0.02	<0.02	<0.02	**4**	8	8	8	2
Ceftazidime	PMZ	500	4	<0.06	<0.06	**2**	4	4	4	4	2
	CPZ	250	4	<0.06	<0.06	<0.06	4	4	4	4	0

Table 5-6 MICs of *bpeAB-oprB* expressing strain, Bt45 in the presence of varying concentrations of the EPIs, PMZ and CPZ. The fold potentiation is the fold difference between MIC of the antibiotic in the absence of EPI and the MIC of the antibiotic in combination with the maximum concentration of EPI which is not inhibitory itself. MICs of the antibiotic when in combination with an EPI at a concentration above the MIC of the EPI alone are not synergistic, but are a reflection of complete inhibition by the EPI itself. Combinations of EPI and antibiotic where synergy is recognized are indicated in boldface.

Antibiotic	EPI	EPI MIC	MIC of antibiotic at indicated concentration of EPI								Fold potentiation
			0	1000	500	250	125	62.5	31.3	15.6	
Gentamicin	PMZ	500	2	<0.03	<0.03	**1**	**1**	**1**	2	2	2
	CPZ	125	2	<0.03	<0.03	<0.03	<0.03	2	2	2	0
Clarithromycin	PMZ	500	4	<0.5	<0.5	4	4	4	4	4	0
	CPZ	125	4	<0.5	<0.5	<0.5	<0.5	4	4	4	0
Clindamycin	PMZ	500	512	<1	<1	**128**	**256**	512	512	512	4
	CPZ	125	512	<1	<1	<1	<1	512	512	512	0
Norfloxacin	PMZ	500	8	<0.02	<0.02	8	8	8	8	8	0
	CPZ	125	8	<0.02	<0.02	<0.02	<0.02	**0.125**	**2**	8	64
Ceftazidime	PMZ	500	4	<0.06	<0.06	4	4	4	4	4	0
	CPZ	125	4	<0.06	<0.06	<0.06	<0.06	4	4	4	0

in strain Bt212. In regard to strain Bt45, PMZ and CPZ were inhibitory alone at concentrations ≥500 and 125 μM, respectively (**Table 5-6**). The concentrations at which PMZ or CPZ alone were inhibitory differed between strains Bt43, Bt44, and Bt45 indicating that at least CPZ was a substrate for both AmrAB-OprA and BpeAB-OprB. With the exception of gentamicin, PMZ did not potentiate the action of any antibiotics at concentrations <250 μM. Likewise, CPZ was only effective at 62.5 μM.

5.5 Discussion

Here we have described the construction of a *B. thailandensis* efflux deficient strain which can be used as a surrogate background for expression of efflux pumps from *B. pseudomallei*. *B. thailandensis* E264 has homologues of *amrAB-oprA* (*BTH_I2445-BTH_I2444-BTH_I2443*) and *bpeAB-oprB* (*BTH_I0680-BTH_I0681-BTH_I0682*) which were deleted, resulting in a *B. thailandensis* E264 isogenic derivative with a double pump deletion, Bt38. It was necessary to delete the *B. thailandensis* homologues of *amrAB-oprA* and *bpeAB-oprB* so that the phenotype of expression of either *amrAB-oprA* or *bpeAB-oprB* from *B. pseudomallei* was not muted or convoluted by expression of the homologues in the surrogate strain.

Use of efflux pump deficient *P. aeruginosa* as a surrogate host for characterizing *B. pseudomallei* efflux pumps was previously described and resulted in identification of *B. pseudomallei* BpeEF-OprC as a trimethoprim efflux pump (14). However, use of a more closely related surrogate strain, such as *B. thailandensis* decreases misinterpretations of substrate/pump interactions due to other factors such as differences in LPS or outer membrane permeability which maybe more pronounced between distantly related species. *B. thailandensis* E264 *BTH_I2445-BTH_I2444-BTH_I2443* (*amrAB-oprA*)

deletion mutants have been described previously, however, these mutants had additional chromosomal mutations which were introduced to facilitate genetic mutation but ultimately interfered with complete characterization of *amrAB-oprA;* whereas Bt36 is an isogenic derivative of *B. thailandensis* E264 (1).

Deletion of *BTH_I2445-BTH_I2444-BTH_I2443* resulted in a pattern of susceptibility similar to that of the *B. pseudomallei amrAB-oprA* mutant described previously (22). The exception to this is the identification of doxycycline as a substrate for this pump; however, the original investigators did not report testing the MIC of doxycycline. Additionally, when *amrAB-oprA* from *B. pseudomallei* was expressed in *B. thailandensis* Bt44, clindamycin, norfloxacin, and tetracycline were all identified as substrates for AmrAB-OprA. These substrates were not previously identified because they are also substrates for BpeAB-OprB; thus, BpeAB-OprB compensates to extrude these substrates in the absence of AmrAB-OprA. Similar compensation has been observed in many other Gram negative bacteria including both *E. coli* and *P. aeruginosa* both of which express efflux pumps with overlapping substrate profiles (15, 31). The finding that AmrAB-OprA has additional substrates which are masked by expression of BpeAB-OprB has been confirmed in *B. pseudomallei* using isogenic mutants similar to those described here for *B. thailandensis* (T. Mima and H.P. Schweizer, unpublished results).

In order to fully assess the phenotype of a Δ(*bpeAB-oprB*) (Δ[*BTH_I0680-BTH_I0681-BTH_I0682*]) mutant in *B. thailandensis*, *amrAB-oprA* had to be deleted first. Like the AcrAB-TolC and MexAB-OprM efflux systems of *E. coli* and *P. aeruginosa,* respectively, AmrAB-OprA and it's homologue in *B. thailandensis* extrude

such a wide variety of substrates and confer such a high level of resistance, that the effect of other pumps is masked unless they are greatly over-expressed or these pumps are repressed or deleted (8, 20, 24). Norfloxacin, tetracycline, doxycycline, and acriflavin were identified as BpeAB-OprB substrates when increased susceptibility to these antibiotics was observed in the *bpeAB-oprB* mutant, Bt34 compared to MICs of the same antibiobtics in the parental strain *B. thailandensis* E264. Additionally, when MICs of the *B. thailandensis* double mutant, Bt38, were compared to the Δ*amrAB-oprA* deletion mutant, Bt36, clarithromycin, clindamycin, and erythromycin were all identified as substrates of the *B. thailandensis* BpeAB-OprB homologue. All the same antibiotics were substrates for BpeAB-OprB when this pump was expressed in the *B. thailandensis* surrogate strain, Bt38. These findings are in contrast to substrates previously identified for BpeAB-OprB, which included only aminoglycosides and macrolides, differing from the published substrates of AmrAB-OprA by the inclusion of spectinomycin and clarithromycin as substrates (3). However, using a different strain background than the original investigators, our lab has identified the same substrate profile for *B. pseudomallei* BpeAB-OprB as is demonstrated here for *B. thailandensis* (T. Mima and H.P. Schweizer, unpublished results).

Once it had been established that the substrate profiles of AmrAB-OprA and BpeAB-OprB were maintained when expressed in the surrogate *B. thailandensis* background, we tested whether previously identified efflux pump inhibitors, PMZ and CPZ would potentiate the action of antimicrobials which were substrates for these pumps. PMZ and CPZ were found to potentiate the action of aminoglycosides, macrolides, β-lactams, and fluoroquinolones against *B. pseudomallei* KHW, presumably by inhibiting

AmrAB-OprA and BpeAB-OprB (and perhaps a hitherto unidentified pump as β-lactams have not been identified as substrates for either of these pumps) (2). We found that PMZ but not CPZ potentiated the action of all substrates of AmrAB-OprA tested. Additionally, neither PMZ nor CPZ affected the action of ceftazidime, which is not a substrate for AmrAB-OprA. Furthermore, CPZ potentiated the action of norfloxacin in strains expressing BpeAB-OprB, but did not affect the MICs of other substrates for this pump. The MIC for clindamycin in strains expressing BpeAB-OprB was decreased by 4 fold in the presence of PMZ.

Inhibitors of RND efflux have several proposed mechanisms of action including (i) inhibition of pump assembly (ii) competitive or non-competitive substrate exclusion or (iii) disruption of the proton gradient which is the energy source for RND transporters (18, 19). The latter is the least desirable as it does not confer selective toxicity however, that is one of the roles that phenothiazines such as PMZ and CPZ are thought to play as EPIs (12, 13) That PMZ and CPZ appear to act specifically to inhibit select substrates of either AmrAB-OprA or BpeAB-OprB supports the supposition by Chan et al. (2) that these EPIs appear to acting in a manner unrelated to the disruption of the proton gradient. Nonetheless, our findings also support that of Chan et al. that both PMZ and CPZ are only effective at relatively high concentrations. Therefore, a search for alternative efflux pump inhibitors with feasible clinical applications is necessary. The panel of *B. thailandensis* strains described here, each expressing one RND efflux operon from *B. pseudomallei* will facilitate such a search. The benefits of such a system over using *B. pseudomallei* for a high through-put screen for EPIs include (i) the advantage of performing such a screen under BSL2 conditions (ii) interactions of EPIs can

automatically be attributed to a specific pump (rather than to efflux in general as in (2)) which will expedite downstream characterization of such a compound (iii) the efflux deficient control strain will aid in identifying compounds which act in a non-specific manner to increase susceptibility, thereby reducing false-positive lead compounds. Lastly, this panel of strains can be easily modified to reflect additional efflux pumps contributing to clinically relevant resistance as such pumps are identified.

5.6 References

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CHAPTER 6

6.1 Concluding remarks

Burkholderia pseudomallei, the etiologic agent of melioidosis, was relatively unheard of in the western world until its prioritization as a category B select agent which led to an increase in basic scientific research on this organism (1). Elucidating the specific mechanisms leading to the characteristic high-level intrinsic resistance of *B. pseudomallei* has been of particular interest (5, 7, 11, 13, 14, 17). This specific goal has dual benefits as the less expensive and more effective treatment options for melioidosis generated as a result of research on antibiotic resistance will be useful in the event of an intentional release of *B. pseudomallei* as well as in endemic regions, such as southeast Asia, where funding and resources for such research is limited.

Antimicrobial resistance as a result of efflux by the RND family of transporters has been well documented as a major contributor to the intrinsic resistance to both macrolides and aminoglycosides in *B. pseudomallei* (7, 13). However, reports of isolates that were susceptible to these antibiotics lead us to question not only the molecular mechanisms of this susceptibility, but also whether the efflux pumps normally responsible for the intrinsic resistance to these antibiotics were consistently expressed in clinical and environmental isolates from the same region as the susceptible isolates. To further assess the contribution of both characterized and putative RND *B. pseudomallei*

efflux pumps to antimicrobial resistance, we sought to correlate efflux pump expression and MICs to clinically relevant antibiotics. Realizing that efflux pumps conferring resistance to otherwise clinically useful antibiotics were widely expressed in both clinical and environmental isolates of *B. pseudomallei* and that inhibition of these pumps would potentially broaden the spectrum of treatment options for melioidosis; we designed a panel of *B. thailandensis* strains serving as a platform for surrogate expression of *B. pseudomallei* efflux pumps (AmrAB-OprA and BpeAB-OprB). This panel of strains can be used to screen small molecule libraries for potential inhibitors of these efflux pumps. The research efforts detailed in this dissertation have made significant contributions to the study of antimicrobial resistance as a result of efflux in *B. pseudomallei* including:

(i) The mechanism of susceptibility to aminoglycosides and macrolides in rare clinical isolates can be attributed to insufficient expression (strains 2188a and 3799a) or deletion (strains 708a) of the efflux pump *amrAB-oprA* (chapter 3). A report by Simpson et al., identified three gentamicin susceptible clinical isolates of *B. pseudomallei* and, based on simultaneous susceptibility to several other aminoglycosides, presumptively attributed the susceptibility seen in these strains to a deficit related to AmrAB-OprA, as this pump was known to confer aminoglycoside and macrolide resistance (13, 16). Using *q*RT-PCR, we demonstrated that these three strains had decreased expression of *amrB*, as compared to that of strain 1026b in which *amrAB-oprA* expression is sufficient to confer resistance to aminoglycosides and macrolides. Furthermore, expression of *amrB* was increased in gentamicin

resistant mutants of strains 2188a and 3799a, as compared to expression in their parental strains. Deletion of *amrAB-oprA* in the mutant strains resulted in susceptibility to gentamicin. Clearly, insufficient expression of *amrAB-oprA* in strains 2188a and 3799a results in susceptibility to aminoglycosides; however, increased expression in the resistant mutants could not be attributed to (i) mutations in the regulatory regions upstream of the *amrAB-oprA* (ii) mutations in *amrR,* the putative repressor of *amrAB-oprA* (iii) or *armR* functioning as an activator. Thus, it is likely that there is a hitherto unidentified mechanism for regulating the expression of *amrAB-oprA.* Interestingly, strain 708a had a large deletion (~131 Kb) on chromosome 1, which included *amrAB-oprA,* thereby leading to the observed susceptibility of this strain to aminoglycosides and macrolides. In addition to *amrAB-oprA* this deletion contains several other gene clusters previously thought to be required for virulence; for example, the genes encoding for the siderophore malleobactin. As strain 708a was fully virulent despite this large chromosomal deletion, the strain may be a useful tool for identifying the genes that provide alternate pathways for processes carried out by the genes in the deletion (i.e. alternate siderophores). Lastly, the characterization of a fully virulent strain containing a natural deletion of *amrAB-oprA* both facilitates and validates the use of Δ*amrAB-oprA* strains for select-agent compliant genetic manipulation experiments. In Thailand the occurrence of such strains is at least 1 in 1,000 isolates (0.1%) among clinical isolates while in Singapore the occurrence is more frequent, 5 in 243 isolates (2%) (10, 16). These

estimates are probably very conservative given the use of Ashdown's agar (which contains gentamicin) for isolation of *B. pseudomallei*.

(ii) The finding that insufficient expression of an RND efflux pump in *B. pseudomallei* lead to exquisite susceptibility to both aminoglycosides and macrolides lead us to question the consistency of expression of RND efflux pumps in *B. pseudomallei* isolates from Thailand. Using *q*RT-PCR, we were able to conclude that expression of seven RND efflux pumps (*amrB, bpeB, bpeF, bpeH, BPSL0309, BPSL1267*, and *BPSL1567*) was prevalent among both clinical and environmental isolates. Furthermore, *bpeH*, a pump whose substrates are unknown, was expressed at significantly higher levels in clinical strains as compared to environmental strains. The significance of this finding remains to be elucidated, perhaps by studying the virulence of strains over expressing *bpeH* as compared to that of *bpeH* deficient strains in an animal model. We were also able to make several correlations between efflux pump expression and resistance (or susceptibility) to clinically significant antibiotics. For example, increasing expression of both *BPSL0309* and *bpeH* could be correlated to increasing resistance to gentamicin. Determination of whether gentamicin is a substrate for either of these pumps or, alternatively, whether these pumps are co-regulated with a pump for which gentamicin is a substrate will require further experimentation. Interestingly, we identified inverse relationships between expression of several pumps (*bpeB, BPSL1567,* and *BPSL0309*) and resistance to co-trimoxazole, ceftazidime, and co-

amoxiclav. That these antibiotics are not substrates for these pumps is definitive, however, further experimentation is required to identify the relationship between expression of these pumps and susceptibly to co-trimoxazole, ceftazidime, and co-amoxiclav. One possibility is that these pumps are inversely co-regulated with the genes encoding resistance mechanism for these antibiotics (such as porins or β-lactamases).

(iii) Inhibition of efflux pumps known to contribute to the intrinsic resistance of *B. pseudomallei* to otherwise effective antimicrobials will broaden the spectrum of treatment options for melioidosis. In order to facilitate screening for such an inhibitor, we developed a panel of *B. thailandensis* strains which surrogatley express *B. pseudomallei* efflux pumps, *amrAB-oprA* and *bpeAB-oprB*. Use of such surrogate strains circumvents working under BSL3 conditions, which is necessary for the select agent *B. pseudomallei* but not its close relative, *B. thailandensis*. The *B. thailandensis* homologues of *amrAB-oprA* and *bpeAB-oprB* were both deleted from *B. thailandensis* E264, resulting in an efflux deficient surrogate strain. *amrAB-oprA* and *bpeAB-oprB* (uncoupled from their native promoters and driven by an inducible promoter) from *B. pseudomallei* were each separately inserted, in single copy, into the chromosome of this efflux deficient surrogate strain. When expressed in the surrogate strain, both AmrAB-OprA and BpeAB-OprB had the same substrate profile as observed in the native (*B. pseudomallei* 1026b) background. Additionally, when expressed in the *B. thailandensis* surrogate strain AmrAB-

OprA and BpeAB-OprB could be inhibited by phenothiazines (a class of drugs whose efflux inhibition properties have been previously described in *B. pseudomallei* (6)), confirming the use of the efflux deficient *B. thailandensis* strain as a surrogate host for screening compound libraries for efflux pump inhibitors. In addition to creating a surrogate host strain to be used for discovery of efflux pump inhibitors, we also found that BpeAB-OprB (both from *B. thailandensis* and *B. pseudomallei*) had a different substrate profile than had been described in the literature previously (7); we identified macrolides, quinolones and tetracyclines as substrates and not aminoglycosides. The same conclusions about the substrates of BpeAB-OprB were drawn upon characterization of this pump in isogenic mutants of *B. pseudomallei* 1026b (T. Mima and H.P. Schweizer, unpublished data).

This work has served to further the understanding of the contribution of RND efflux to antimicrobial resistance in *B. pseudomallei.* Additionally, we have created an important tool that can be used as a platform for efflux pump inhibitor discovery which will facilitate development of improved treatment options for melioidosis. However, several unanswered questions still exist. It may be prudent to attempt to assess the rate of isolation of gentamicin susceptible *B. pseudomallei* strains from the environment in order to estimate how often these strains may be missed using current diagnostic techniques which rely on gentamicin resistance (3). Early diagnosis is especially important in the treatment of melioidosis and if gentamicin susceptibility is occurring more frequently than currently realized, development of alternative diagnostics may be required (8).

We know that efflux pumps, both putative and characterized, are widely expressed in clinical and environmental isolates from Australia and Thailand, however, without identifying substrates for the putative pumps we will be unable to accurately assess the potential roles of these pumps, both physiological and in terms of antibiotic resistance (12). Characterization of efflux pumps using *Pseudomonas aeruginosa* as a surrogate host strain has been described (11). However, in addition to its potential role in efflux pump inhibitor discovery, the efflux pump deficient *B. thailandensis* strain described in chapter 3 provides an improvement over the *P. aeruginosa* surrogate strain for surrogate characterization of putative *B. pseudomallei* efflux pumps, as it is more closely related to *B. pseudomallei* (11).

The regulatory mechanisms governing expression of the "resistome" have not been well characterized in *B. pseudomallei* but, based on knowledge from other bacteria that display high-level intrinsic resistance to antibiotics, we can assume that regulation is multifaceted and complex (2, 4, 9, 15). The observations that (i) *amrAB-oprA* appears to be regulated by some mechanism other than AmrR (ii) that expression of *bpeH* and *BPSL0309* can be correlated to increased resistance to gentamicin (although it is unlikely that gentamicin is a substrate for these pumps, given the susceptibly observed in Δ*amrAB-oprA* mutants) and (iii) an inverse correlation exists between expression of *bpeB, BPSL1567,* and *BPSL0309* and resistance to co-trimoxazole, ceftazidime, and co-amoxiclav all hint to global regulation of RND efflux in *B. pseudomallei.* Elucidation of such global regulation mechanisms may lead to identification of new drug targets, facilitating our ultimate objective of developing improved treatment options for melioidosis.

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